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13. ABSTRACT ***(Maximum 200 Words)***

NKX3.1 is a prostate-specific homeobox gene that maps to chromosome 8p21, a locus frequently deleted in prostate cancer. In the mouse, *Nkx3.1* controls differentiated functions and limits growth of prostate epithelial cells. Although *NKX3.1* may be a tumor suppressor in humans, no cancer specific mutations have been identified in human prostate cancer. Previously, a C→T polymorphism at nucleotide 154 of *NKX3.1* was identified, resulting in alteration of codon 52 from Arg→Cys in 10-14% of the population. The effects of the NKX3.1 polymorphism were examined by identifying the consensus DNA binding sites for wild-type (WT) and polymorphic (R52C) NKX3.1. Binding specificity was confirmed by gel mobility shift. Both WT and R52C NKX3.1 specifically repressed transcription from a reporter gene downstream from multiple copies of the NKX3.1 binding site. Amino acid 52 is adjacent to a putative PKC phosphorylation site at serine 48 of NKX3.1. *In vivo* analysis of NKX3.1 indicated that the protein is phosphorylated at serine. *In vivo and in vitro* phosphorylation studies indicated that R52C NKX3.1 phosphorylation was reduced relative to WT NKX3.1. Phosphorylation of WT NKX3.1 resulted in decreased DNA binding affinity, while R52C NKX3.1 binding affinity appeared unaltered, presumably due to repressed phosphorylation of the mutant protein. The data suggested that polymorphism at position 52 may alter kinase regulation of NKX3.1.

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INTRODUCTION

We have made substantial progress on this project in the past year. The results of this work have yielded one published manuscript and one manuscript that will be submitted for publication. *Due to significant duplication, figures are not included in the Body, but are shown in the manuscripts included in the Appendix.*

NKX3.1 is a prostate-specific homeoprotein that suppresses growth and activates differentiated functions in prostatic epithelial cells. The hypothesis of this project is that decreased expression of NKX3.1 in human prostate cancer contributes to pathogenesis and to tumor progression. Moreover, a mutation in *NKX3.1*, present in 10% of the population, may be a risk factor for prostate cancer. *NKX3.1* is a homeobox gene with prostate-specific expression in the adult (1). *NKX3.1* was a candidate target gene for disruption by the common 8p21 chromosomal deletion in prostate cancer. However, mutational analysis failed to find any tumor-specific mutations of *NKX3.1* in human prostate cancer tissues (2). Gene targeting studies showed that *NKX3.1* had potent growth suppressing and differentiating effects on prostatic epithelium and that even mice heterozygous for targeted disruption of *Nkx3.1* had dysplastic prostate morphology (3). This suggested that gene dosage and, therefore, the amount of protein may be important for the growth suppression and differentiation effects of NKX3.1.

We showed that NKX3.1 protein expression was lost in approximately 40% of human prostate cancer specimens (Bowen et al, in press). To accomplish this work we derived purified recombinant NKX3.1 protein and generated a high-titer rabbit antiserum to NKX3.1. The antiserum is useful for immunohistochemical, western blotting and immunoprecipitation. We analyzed NKX3.1 expression in 61 human prostate specimens, 30 of which contained prostate cancer as well as nonmalignant prostatic epithelium. One hundred percent of normal prostatic glands expressed NKX3.1, but only 57% (17/30) of the cancers had expression. We also completed an immunohistochemical survey of 17 normal human tissues to show that NKX3.1 was expressed in testis (as previously suggested by mRNA analysis), in rare pulmonary mucous glands, and in isolated islands of transitional epithelium of the ureter, but in no other tissues. Significantly, there was no expression of NKX3.1 in human bone marrow, suggesting that NKX3.1 may be an immunodetection marker for micrometastatic prostate cancer.

Mutational analysis of *NKX3.1* showed that the DNA sequence of the coding region was intact in human prostate cancer (2). We discovered a transition mutation in several tissues that changed a C $\rightarrow$ T at nucleotide 154, resulting in a codon change from arginine to cysteine at amino acid 52 (R52C). Using a Taqman genotyping assay we showed that the R52C mutation is present in approximately 10% of the population and nearly equally represented in Caucasians and African Americans. Amino acid 52 is part of a consensus protein kinase C phosphorylation sequence directing phosphorylation of serine-48. Site-directed mutagenesis of serine-48 to alanine abrogated phosphorylation. R52C diminished phosphorylation of serine-48 by 80%. Using oligonucleotide selection with recombinant NKX3.1 protein we identified the NKX3.1 DNA recognition sequence as TAAGTA. Phosphorylation of wild-type NKX3.1 inhibited binding to TAAGTA by 90%, but binding of R52C NKX3.1 to TAAGTA was not affected by phosphorylation, suggesting that R52C NKX3.1 has different DNA binding properties compared to wild-type protein. To determine if there is a phenotype for R52C, we are in the midst of genotyping a cohort of 1200 prostate cancer patients and controls from the Physicians Health Study to determine if NKX3.1 R52C is a risk fa[1]ctor for prostate cancer.

BODY

Aim 1. Identification of the NKX3.1 DNA Binding Domain.

A. Determination of the NKX3.1 Consensus Binding Sequence by SAAB Assay

The SAAB assay originally described by Blackwell and Weintraub (4) with modifications by Chen and Schwartz (5) was used to determine a consensus DNA binding sequence for bacterially expressed WT and R52C NKX3.1 fusion proteins. Purified proteins were used to select preferred binding sequences from a random pool of double-stranded DNA. Five cycles of binding selection were used to isolate optimal binding sequences and the enriched double-stranded DNA was cloned and sequenced. Alignment of the 20 selected sequences each for WT and R52C NKX3.1 revealed an identical consensus sequence of 6 nucleotides (TAAGTA) with an individual nucleotide frequency of occurrence ranging from 78 to 100 %. The NKX3.1 consensus sequence is similar, but not identical to the binding sequences of other NK-2 class homeoproteins such as the CAAGTG site for Nkx2.1 (6) and TNAAGTG for Nkx2.5 (5). The consensus sequence for NKX3.1 is also similar to the TAAGTG consensus binding sites of human Hox11 (7) and salmon Isl-2 (8), members of the Hox and LIM families of homeoproteins, respectively. The selected sequences also contained the TAAT motif recognized by a majority of homeodomain proteins with a characterized DNA binding site (9). The TAAT motif was present at least once in 43 % of the combined WT and R52C selected sequences. In a similar binding assay, Nkx2.5 also selected sequences containing TAAT in addition to the consensus TNAAGTG binding site (5,10).

Specific binding of WT and R52C NKX3.1 to the TAAGTA site was demonstrated by gel mobility shift. Maltose binding protein (MBP) lacking the NKX3.1 moiety did not bind the TAAGTA sequence, suggesting that protein-DNA interactions were specifically due to the NKX3.1 segment of the fusion protein. Binding specificity was confirmed by competition with a 10-fold molar excess of unlabeled NKX3.1 consensus sequence, which competed for the TAAGTA probe. An Oct POU-1 homeodomain binding site was included as a control for nonspecific DNA binding. The Oct site contains a CTAAAC binding site rather than the preferentially bound TAAGTA sequence. Neither protein bound the Oct probe. All sequences selected by WT and R52C NKX3.1 in the SAAB assay exhibited a high frequency of A/T-rich nucleotides flanking the TAAGTA binding site. However, when TAAGTA sequences were flanked with hexanucleotide GC-sequences at either the 5' or 3' end, no effect on NKX3.1 binding was seen (data not shown). It is possible that the high frequency of A/T base pairs flanking the NKX3.1 consensus site was an artifact of the binding assay. In fact, previous selection assays using NK-class homeoproteins also yielded sequences with a relatively high A/T content outside of their respective consensus binding sites (5,11).

B. WT and R52C NKX3.1 Exhibit Similar Binding Affinities

To determine if the polymorphism at position 52 affected DNA binding affinity, the dissociation constants for WT and R52C NKX3.1 binding to the TAAGTA consensus site were measured. Increasing amounts of protein were incubated with a constant amount of radiolabeled probe. Following quantitative analysis, the data was plotted by the method of Scatchard (15) for binding constant determination. The dissociation constants for the proteins were nearly identical, exhibiting K_d values of 2.3 nM for WT and 2.6 nM for R52C NKX3.1. These values are comparable to the dissociation constants for the Nkx2.1 (3.0 nM), Antennapedia (1.6 nM), and Engrailed (1-2 nM) homeoproteins (13,16). The results indicate that mutation at position 52 of NKX3.1, which lies N-terminally outside of the homeodomain region, did not affect DNA binding affinity.

C. NKX3.1 Acts as a Transcriptional Repressor

Luciferase reporter vectors were constructed with 3 tandem NKX3.1 binding sites in the sense (TAAGTA-TK-Luc) and antisense (ATGAAT-TK-Luc) directions upstream from a Herpes simplex virus TK promoter. Cotransfection of TSU-Pr1 cells with TAAGTA-TK-Luc and an NKX3.1 expression vector resulted in decreased transcription compared with control cells cotransfected with TAAGTA-TK-Luc and empty expression vector, suggesting that in this context NKX3.1 behaved as a transcriptional repressor. There was no apparent difference in the relative luciferase activities of WT and R52C NKX3.1 cotransfected with TAAGTA-TK-Luc, suggesting that the polymorphism did not affect NKX3.1 transcriptional repression *in vitro*. Previous binding studies have shown that a murine Nkx3.1 homeodomain will not bind to a CACGTG sequence (14). When the NKX3.1 binding sites were mutated to CACGTG, the relative luciferase activity using NKX3.1 expression plasmid had little effect on transcription, indicating that transcriptional repression with reporter constructs containing 3 copies of TAAGTA was due to specific binding of NKX3.1. The level of transcriptional repression using ATGAAT-TK-Luc and WT NKX3.1 expression vector was similar to that observed using TAAGTA-TK-Luc and WT expression vector, indicating that the orientation of the TAAGTA sequence does not affect transcriptional repression due to NKX3.1 binding. Insertion of the TAAGTA and CACGTG inserts increased luciferase activity relative to the unmodified TK-Luc reporter plasmid. Based on these results, it is likely that these inserts contain binding sites for transcriptional activators endogenously expressed in TSU-Pr1 cells. For example, the TAAGTA insert contains multiple sequences of ATTA (complement of TAAT), which is bound by most homeoproteins (9,17). Also, the CACGTG sequence is a high affinity MYC binding site (17).

Aim 2. Interaction of NKX3.1 and AR on MMTV LTR.

We had presented preliminary data showing that NKX3.1 wild type and polymorphic proteins inhibited transcription initiated from the promoter in the murine mammary tumor virus long terminal repeat (MMTV LTR). MMTV LTR contains a steroid response element that binds the androgen receptor (AR) and glucocorticoid receptor (GR). We had hypothesized that four putative NKX3.1 DNA binding domains near the steroid response element were responsible for transcriptional repression by NKX3.1. However, we generated a deletion construct of the MMTV-LTR that removed approximately half of the LTR. The 3' half of the LTR that retained all the putative NKX3.1 binding sites retained no suppressor activity. All the NKX3.1 suppressor binding sites were located in the 5' half of the LTR, more than 300 nucleotides from the steroid hormone response element. This experiment showed that the suppression of MMTV-LTR transcription by NKX3.1 was complex. We may use alternative methods to study the effect of NKX3.1 on AR-driven transcription, which we are currently developing.

Aim 3. Determination of the Role of Amino Acid 52 in Phosphorylation of NKX3.1 by PKC

A. NKX3.1 is Phosphorylated In Vivo.

To determine if NKX3.1 is phosphorylated *in vivo*, TSU-Pr1 cells were transfected with a wild-type NKX3.1 expression construct or empty vector. The NKX3.1 construct contained cDNA for expression of amino acids 1-184 including the N-terminus and homeodomain of NKX3.1. Our unpublished data indicated that expression of transfected NKX3.1 was signinficantly enhanced in TSU-Pr1 and LNCaP cells by deletion of amino acids 185-234. Treatment of TSU-Pr1 cells transfected with the wild-type NKX3.1 construct using [^{32}P]orthophosphate followed by immunoprecipitation with an affinity purified anti-NKX3.1 antiserum detected phosphorylated protein that migrated at approximately 25 kDa. Cells transfected with empty vector or cell lysates immunoprecipitated with pre-immune serum did not yield the 25 kDa band

when treated with [^{32}P]orthophosphate. This band corresponds to the predicted 23 kDa molecular weight of amino acids 1-184 of NKX3.1. As a control for antibody specificity, parallel cultures of TSU-Pr1 transient transfectants were prepared for western blotting. The antibody used for immunoprecipitation was used as primary antibody in the western analysis. No NKX3.1 protein was detected in lysates transfected with the empty vector. The protein detected in lysates transfected with NKX3.1 expression vector also migrated at approximately 25 kDa. NKX3.1 expression is upregulated in LNCaP cells treated with androgen. Endogenously expressed NKX3.1 from LNCaP cells treated with R1881 was also phosphorylated *in vivo* and the level of phosphoprotein was increased by the presence of 100 nM TPA, suggesting that NKX3.1 is phosphorylated *in vivo* by a TPA-induced kinase such as PKC. Treatment of the cells with TPA did not cause an increase in the level of NKX3.1 protein as determined by Western blotting. Phosphoamino acid analysis of the radiolabeled endogenous protein indicated that NKX3.1 is phosphorylated only at serine. Similar phosphoamino acid analysis results were obtained with exogenously labeled NKX3.1 from TSU-Pr1 cells (data not shown).

B. Wild-Type and Mutant NKX3.1 are Differentially Phosphorylated by PKC.

The software program Phosphobase v2.0 (24) was used to analyze the NKX3.1 amino acid sequence for possible phosphorylation sites. Of the consensus sites identified, Ser48 was a candidate phosphorylation site for CaMII, PKA and PKC. This site was of interest because it is located in close proximity to amino acid 52, the site of the NKX3.1 polymorphism. To determine if NKX3.1 could also be phosphorylated by PKC, purified wild-type fusion protein was incubated with [γ-^{32}P]ATP in the presence of a purified mixture of the α, β and γ isoforms of PKC. The results demonstrate that wild-type NKX3.1 fusion protein was phosphorylated *in vitro* by PKC. In addition to NKX3.1 phosphorylation, a minor level of PKC autophosphorylation was present, represented by the 80 kDa band. Phosphobase v2.0 also identified four consensus PKC sites in the amino acid sequence of the maltose-binding protein affinity tag. However, PKC did not phosphorylate maltose-binding protein alone, suggesting that phosphorylation of the fusion protein was specific for the NKX3.1 moiety.

To determine if the polymorphism could affect phosphorylation of NKX3.1, R52C fusion protein was also used as a substrate in a PKC kinase assay. The results indicated that PKC phosphorylation of R52C NKX3.1 was noticeably decreased relative to phosphorylation of wild-type NKX3.1. To ensure that equal amounts of the proteins were loaded onto the gel, samples were prepared in parallel without [γ-^{32}P]ATP. The samples were electrophoresed and blotted with an anti-maltose binding protein antiserum. The results shown in the bottom panel of indicate that equal amounts of fusion protein with either wild-type or R52C NKX3.1 were loaded onto the gel.

To verify that the mutation at position 52 negatively affected phosphorylation, synthetic peptide substrates representing amino acids 43-54 of NKX3.1 were used in PKC kinase assays. The results of the kinase assays using the peptide substrates shown in were consistent with PKC phosphorylation of the full-length proteins. Relative phosphorylation of peptide P-WT representing wild-type NKX3.1 was 3-fold higher than peptide P-R52C representing R52C NKX3.1. Phosphorylation of peptide P-S48A with Ser replaced by Ala at the relative position 48 of NKX3.1 was decreased 33-fold relative to peptide P-WT, indicating that phosphorylation was specific for Ser48. To determine if the effects of the R52C mutation *in vivo*, wild-type, R52C or S48A NKX3.1 mammalian expression vectors were used to transfect LNCaP cells. The R52C mutation decreased phosphorylation of NKX3.1 by 2-fold. Mutating Ser48 to Ala significantly decreased the level of *in vivo* phosphorylation of NKX3.1, providing evidence that Ser48 is a phosphoacceptor.

C. Phosphorylation by PKC Represses Wild-Type but not R52C NKX3.1 DNA Binding.

The effects of PKC phosphorylation on NKX3.1 DNA binding were determined by gel shift assays. Purified NKX3.1 fusion proteins were treated with PKC in the presence or absence of ATP and the proteins

were included in gel shift assays with a radiolabeled NKX3.1 consensus DNA binding sequence. Phosphorylation of wild-type NKX3.1 decreased the apparent binding affinity of the protein for the consensus sequence by 3-fold relative to the nonphosphorylated protein. However, the apparent DNA binding affinity of R52C was not noticeably altered upon treatment of PKC in the presence of ATP. To determine if Ser48 of NKX3.1 was directly involved in modulating DNA binding affinity due to PKC phosphorylation, fusion protein with Ser48 mutated to Ala (S48A) was included in a gel shift assay. Replacing Ser with Ala disrupted phosphorylation-regulated DNA binding, indicating that the phosphorylation state of Ser48 modulates NKX3.1 DNA binding affinity. For controls, the two other consensus PKC phosphorylation sites at Thr119 and Thr179 were mutated to Ala. PKC treatment of NKX3.1 with T119A yielded results that were similar to wild-type NKX3.1. Interestingly, mutating Thr179, which is in the homeodomain, to Ala abolished NKX3.1 DNA binding, regardless of protein phosphorylation.

D. R52C Polymorphism Affects Androgen-Regulated Reporter Gene Expression.

The MMTV-LTR has an androgen response element (ARE) that mediates transcriptional activation by the androgen receptor. The ARE is located 449 nucleotides from the 3'-end of the LTR and 151 nucleotides upstream of the TATA box. Expression of the reporter construct was activated by the addition of an AR expression plasmid in the presence of 1 nM DHT. Cotransfection of wild-type NKX3.1 or NKX3.1 R52C expression plasmid with the reporter vector in the presence of DHT decreased reporter gene expression in proportion to the amount of NKX3.1 plasmid added. These results are consistent with our previous data indicating that NKX3.1 could repress transcription of luciferase from a reporter vector with a thymidine kinase promoter with tandem upstream TAAGTA binding sites. Co-transfection of NKX3.1 R52C expression plasmid enhanced transcriptional repression of the luciferase reporter gene by 115–120 % relative to wild-type NKX3.1. These results are in agreement with the idea that the R52C polymorphism decreases NKX3.1 phosphorylation, resulting in deregulation of NKX3.1 DNA binding affinity.

E. Prevalence of R52C Polymorphism.

To determine the frequency that the NKX3.1 R52C polymorphism occurred in the population and to carry out a preliminary investigation of whether NKX3.1 R52C affected risk for prostate cancer, we analyzed germ line DNA from age-matched Caucasian and African American cases and controls in a cohort study of prostate cancer. Overall, 13% of men in the study were found to carry the NKX3.1 R52C polymorphism. There was a somewhat higher prevalence of the polymorphism among Caucasians than African Americans. In this small sample there was no indication that cancer risk was conferred by NKX3.1 R52C.

Key Research Accomplishments

- Determination and characterization of the NKX3.1 DNA binding site
- Characterization of wild-type and R52C NKX3.1 binding to the NKX3.1 DNA consensus site
- Determination of the in vivo transcriptional function of NKX3.1
- Determination of in vivo phosphorylation of NKX3.1
- Analysis of the effects of the R52C polymorphism on NKX3.1 phosphorylation

Reportable Outcomes

At present, the results described herein have yielded one published manuscript:

Steadman, D. J., Giuffrida, D., and Gelmann, E. P. DNA Binding Sequence of the Human Prostate-Specific Homeodomain Protein NKX3.1. *Nuc. Acids Res. 28, 2389-2395* (2000)

A second manuscript is to be submitted:

Steadman, D. J., Swope, S., Voeller, H. J., and Gelmann, E. P. Phosphorylation of NKX3.1, *Manuscript In Preparation*

The results have also been published as an abstract for the 2000 AACR meeting:

Steadman, D. J., Bowen, C., Voeller, H. J., and Gelmann, E. P. Immunohistochemical and Biochemical Characterization of NKX3.1 in Normal Human Tissues and Prostate Cancer. *Proc. Amer. Assoc. Cancer Res. 41*, 667 (2000).

Conclusions

The following can be concluded from the work performed to date:

1) Wild-type and R52C NKX3.1 optimally bind a TAAGTA DNA sequence
2) Wild-type and R52C NKX3.1 bind to the TAAGTA sequence with similar affinities
3) Wild-type and polymorphic NKX3.1 repress transcription of a reporter gene with three upstream TAAGTA binding sites
4) Wild-type NKX3.1 is phosphorylated *in vivo* and only at serine
5) R52C NKX3.1 phosphorylation by PKC is significantly reduced relative to wild-type NKX3.1
6) Phosphorylation of wild-type NKX3.1 reduces DNA binding affinitly, however phosphorylation of R52C NKX3.1 does not noticeably affect R52C DNA binding
7) Phosphorylation state of Ser48 modulates NKX3.1 DNA binding
8) R52C polymorphism affects NKX3.1 repression of androgen receptor driven transcription

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DNA-binding sequence of the human prostate-specific homeodomain protein NKX3.1

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ABSTRACT

NKX3.1 is a member of the NK class of homeodomain proteins and is most closely related to *Drosophila* NK-3. NKX3.1 has predominantly prostate-specific expression in the adult human. Previous studies suggested that NKX3.1 exerts a growth-suppressive effect on prostatic epithelial cells and controls differentiated glandular functions. Using a binding site selection assay with recombinant NKX3.1 protein we identified a TAAGTA consensus binding sequence that has not been reported for any other NK class homeoprotein. By electromobility shift assay we demonstrated that NKX3.1 preferentially binds the TAAGTA sequence rather than the binding site for Nkx2.1 (CAAGTG) or Msx1 (TAATTG). Using mutated binding sites in competitive gel shift assays, we analyzed the nucleotides in the TAAGTA consensus sequence that are important for NKX3.1 binding. The consensus binding site of a naturally occurring polymorphic NKX3.1 protein with arginine replaced by cysteine at position 52 was identical to the wild-type binding sequence. The binding affinities of wild-type and polymorphic NKX3.1 for the TAAGTA consensus site were very similar, with values of 20 and 22 nM, respectively. Wild-type and polymorphic NKX3.1 specifically repressed transcription of luciferase from a reporter vector with three copies of the NKX3.1-binding site upstream from a thymidine kinase promoter. The data show that among NK family proteins NKX3.1 binds a novel DNA sequence and can behave as an *in vitro* transcriptional repressor.

INTRODUCTION

Homeobox genes encode proteins that contain highly conserved DNA-binding regions called homeodomains. Homeodomain proteins, or homeoproteins, bind to specific DNA sequences and function as transcription factors that regulate eukaryotic development with spatial and temporal specificity (1). NKX3.1 is a novel homeoprotein that belongs to the *Drosophila* NK class of homeodomain proteins. NKX3.1 is most closely related to *Drosophila* NK-3 by virtue of 78% sequence homology to the NK-3 homeodomain region (2–4). Murine Nkx3.1 has 100% sequence homology to the human NKX3.1 homeodomain region and shares 90% sequence homology overall (3–6).

Expression of murine *Nkx3.1* in the developing embryo and fetus has been detected in a variety of tissue types, including mesoderm, vascular smooth muscle, epithelium and regions of the central nervous system (7–9). However, in the adult mouse *Nkx3.1* expression is androgen regulated (5) and restricted primarily to the prostate and the bulbourethral gland (6,9). *NKX3.1* expression in the adult human is also androgen regulated and localized predominantly in the prostate, with low levels also detected in the testis (3,4).

NKX3.1 has been mapped to human chromosome 8p21, a locus that is frequently deleted in prostate cancer (10–13). Therefore, *NKX3.1* is a candidate tumor suppressor gene. However, no cancer-specific mutations of the *NKX3.1* coding region were found in human prostate cancer samples (10). Nevertheless, the potential for NKX3.1 to exert a differentiating and growth suppressing effect on prostatic epithelium was confirmed by targeted gene disruption of *Nkx3.1* in mice (9). Deletion of either one or both copies of *Nkx3.1* resulted in prostatic epithelial hyperplasia and dysplasia that increased in severity with age. Homozygous deletion of *Nkx3.1* caused defective prostate ductal morphogenesis and decreased seminal protein production. Loss of NKX3.1 protein expression may be important in prostate cancer pathogenesis. We have recently shown that ~40% of human prostate cancer samples had diminished expression of NKX3.1 compared to adjacent normal epithelium (Bowen *et al.*, submitted for publication).

Genetic analysis indicated that ~10–15% of human DNA samples contain a polymorphic *NKX3.1* gene characterized by the presence of a C→T polymorphism at nucleotide 154 (10; our unpublished data). The polymorphism resulted in the substitution of arginine by cysteine at codon 52, located N-terminal to the homeodomain. To date, a phenotype has not been identified for the polymorphic protein.

NK-2 class homeoproteins preferentially bind DNA sequences with a CAAG core sequence (14,15), while other NK class homeoproteins preferentially bind TAAT core sequences (16,17). The optimal DNA binding site for *Drosophila* NK-3 or its mammalian homologs has yet to be defined. Using a binding site selection assay, we identified the *in vitro* DNA binding site of NKX3.1. Electromobility gel shift assays were used to analyze binding affinity and nucleotides in the consensus site that are important for NKX3.1 binding. By

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inserting three copies of the NKX3.1-binding site into a luciferase reporter vector, we investigated the transcriptional function of NKX3.1 using reporter gene analysis. Finally, the DNA binding and transcriptional activities of wild-type and polymorphic (R52C) NKX3.1 were compared to determine if differences existed between the proteins.

MATERIALS AND METHODS

Expression and reporter plasmid construction

Full-length NKX3.1 cDNA was synthesized by PCR amplification of full-length wild-type human NKX3.1 cDNA obtained from a normal prostate cDNA library (3). Amplification was carried out for 30 cycles (94°C for 30 s, 62°C for 30 s and 72°C for 60 s) using a Perkin Elmer temperature cycler (Perkin Elmer, Norwalk, CT). The primers (Gibco BRL, Rockville, MD) used in the amplification incorporated *Eco*RI and *Xho*I restriction sites for directional cloning of the amplified DNA into expression vectors. The forward PCR primer has the sequence 5′-CGG-GATCCGAATTCATGCTCAGGGTTCCGGAGCCGC-3′ and the reverse primer has the sequence 5′-GGGCCTCGAGT-CTAGAGTTACCCAAAAGCTGGGCTCCA-3′. The amplified DNA was cloned into a pCRII-Topo vector (Invitrogen, Carlsbad, CA), following the protocol provided by the manufacturer. The DNA was digested with *Eco*RI and *Xho*I restriction enzymes and the fragment representing NKX3.1 cDNA was excised from a 1% agarose gel. The DNA was purified using a QiaexII DNA isolation kit (Qiagen, Valencia, CA) and the resulting fragment was directionally cloned into the *Eco*RI and *Xho*I restriction sites of plasmid pcDNA3 (Invitrogen) for mammalian protein expression. NKX3.1 cDNA was also cloned into the *Eco*RI and *Sal*I sites of the pMAL-C2G vector (New England Biolabs, Beverly, MA) for bacterial protein expression. Wild-type NKX3.1 cDNA was altered using a Stratagene Quikchange Site-Directed Mutagenesis kit following the manufacturer's protocol (Stratagene, La Jolla, CA). R52C NKX3.1 was generated by mutating the nucleotide sequence of codon 52 of wild-type NKX3.1 cDNA from CGC to TGC, thereby changing the corresponding amino acid from arginine to cysteine. The mutant NKX3.1 cDNA was sequenced entirely to ensure that no additional mutations were generated.

Luciferase reporter vectors were constructed by ligating an insert with partial *Xho*I ends containing three copies of TAAGTA or CACGTG into the *Xho*I site of pT109, which contains a herpes simplex virus thymidine kinase (TK) promoter upstream of a firefly luciferase gene. The sequence of one strand of the TAAGTA insert (binding sites underlined) containing a partial *Xho*I site was 5′-TCGATATTAAGTAT-AGGATTAAGTATAGGGATTAAGTAT-3′ and the sequence of one strand of the CACGTG insert was 5′- TCG-ATATCACGTGTAGGATCACGTGTAGGGATCACGTGT-3′. The orientation of the TAAGTA insert was confirmed by sequence analysis.

Bacterial protein expression and purification of recombinant NKX3.1

Plasmids containing wild-type or R52C NKX3.1 cDNA inserted into pMAL-C2G were used to transform competent *Escherichia coli* strain BL21 using standard techniques. Transformants were used to inoculate 10 ml of Luria Bertani (LB) medium and the cultures were incubated with shaking at 220 r.p.m. overnight at 37°C. This culture was used to inoculate 1 l of LB medium containing 0.2% glucose, followed by incubation at 37°C with shaking at 220 r.p.m. until the optical density at 600 nm reached 0.6. Isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 0.3 mM and the culture was incubated for an additional 60 min. Cells were harvested by centrifugation and frozen at –80°C. Approximately 1 g of frozen cells were thawed on ice and resuspended in 10 ml of buffer A [20 mM Tris–HCl, 200 mM NaCl, 1 mM EDTA and 1 mM dithiothreitol (DTT)]. Suspended cells were lysed by sonication and a cell-free extract was prepared by centrifugation. The cell-free extract was loaded onto an amylose column (New England Biolabs) and washed with buffer A. NKX3.1 fusion proteins were eluted from the column with buffer A containing 10 mM maltose. Protein purity was demonstrated by 12% SDS–PAGE and purified protein concentration was determined by the Bradford assay (18).

Selection and amplification binding assay

A selection and amplification binding (SAAB) assay was performed essentially as described for murine Nkx2.5 (15), with modifications. Briefly, 0.5 pmol of a 15 bp random sequence flanked by 20 bp regions of non-random sequence was radiolabeled with 10 μCi of [γ-^{32}P]ATP using T4 polynucleotide kinase. The radiolabeled probe was incubated with 25 pmol of purified wild-type or R52C NKX3.1 fusion protein in binding buffer [10 mM Tris–HCl, pH 7.5, 50 mM NaCl, 7.5 mM $MgCl_2$, 1 mM EDTA, 5% glycerol, 5% sucrose, 0.1% Nonidet P-40, 0.1 μg BSA, 1 μg poly(dI·dC) and 5 mM DTT]. Unbound probe was separated from protein-bound DNA by native 8% PAGE in 0.5× TBE buffer. Protein-bound DNA was detected by autoradiography and the bands representing protein–DNA complexes were excised from the gel. The DNA was eluted from the gel slice overnight at 37°C in elution buffer (0.5 M ammonium acetate, 1 mM EDTA and 0.1% SDS). Eluted DNA was PCR amplified using primers complementary to the 20 bp non-random flanking sequences and purified by native 12% PAGE in 1× TBE buffer. The purified DNA was radiolabeled and used as a probe for another round of SAAB for a total of five rounds. The amount of protein was reduced to 5 pmol for the fourth and fifth rounds of SAAB. Following the final PCR amplification and gel purification, the 55 bp fragment was cloned into pCRII-TOPO using a TOPO-TA cloning kit (Stratagene). The inserts were sequenced on an ABI 377 Nucleotide Sequencer (Perkin Elmer).

Electromobility gel shift assay

Double-stranded DNA used as probe or competitor was prepared by annealing equimolar amounts of complementary oligonucleotides with flanking partial *Bam*HI ends and filled-in with Klenow polymerase. The double-stranded DNA used as probe was radiolabeled with [γ-^{32}P]ATP using T4 polynucleotide kinase. The labeled probe (1 pmol) was added to wild-type or R52C NKX3.1 (125 ng) in binding buffer to a final volume of 20 μl. Competition binding experiments were performed essentially as described by Amendt *et al.* (19) by including unlabeled competitor DNA concentrations of 50, 250 and 500 nM. Competitor DNAs were preincubated with protein at room temperature for 30 min prior to addition of radiolabeled

probe. The nucleotide sequence of one competitor strand is listed, where used, in the appropriate figure. For dissociation constant (K_d) measurements, 1×10^{-10} M radiolabeled probe was incubated with protein concentrations ranging from 0.5 to 200×10^{-9} M for 1 h prior to electrophoresis. Protein–DNA complexes were separated from unbound probe by native 8% PAGE at 175 V for 1 h at ambient temperature in 0.5× TBE buffer following pre-electrophoresis of the gel at 200 V for 60 min. Following electrophoresis, a Molecular Dynamics PhosphorImager Screen (Molecular Dynamics, Sunnyvale, CA) was exposed to the dried gel. A Molecular Dynamics PhosphorImager and ImageQuaNT software (Molecular Dynamics) were used in the quantitative analyses. Binding competition was determined by calculating the ratio of bound to free probe normalized to the absence of competitor DNA. Dissociation constants were calculated from the binding data by plotting bound protein versus free protein and using the expression K_d = [D][P]/[DP] in a non-linear least squares analysis of the data. Free DNA is represented by [D], [P] is the free protein concentration and [DP] is the concentration of DNA–protein complex. Competition binding and dissociation constant measurements were performed at least three times and representative gel shift assays from the competition binding experiments are shown.

Cell culture and reporter gene assays

The prostate carcinoma cell line TSU-Prl (20) was cultured in Improved Minimal Essential Medium (Gibco BRL) supplemented with 5% fetal calf serum. Cells were transiently transfected using 6×10^4 cells/well in a 24-well plate with 250 ng reporter plasmid, 250 ng expression plasmid and 1 ng CMV-*Renilla* plasmid in the presence of 2 μg LipofectAMINE 2000 (Gibco BRL) following the manufacturer's protocol. Cells were lysed 48 h after transfection and the lysate was assayed for firefly and *Renilla* luciferase activities using Dual Luciferase Reporter Assay reagents (Promega, Madison, WI). The data represent the average of three separate experiments normalized by *Renilla* luciferase activity.

RESULTS

Determination of the NKX3.1 consensus binding sequence by SAAB assay

The SAAB assay originally described by Blackwell and Weintraub (21) with modifications by Chen and Schwartz (15) was used to determine a consensus DNA binding sequence for bacterially expressed wild-type and R52C NKX3.1 fusion proteins. Purified proteins were used to select preferred binding sequences from a random pool of double-stranded DNA. Five cycles of binding selection were used to isolate optimal binding sequences and the enriched double-stranded DNA was cloned and sequenced. Alignment of the 20 selected sequences each for wild-type (Fig. 1A) and R52C NKX3.1 (Fig. 1B) revealed an identical consensus sequence of 6 nt (TAAGTA) with an individual nucleotide frequency of occurrence ranging from 78 to 100% (Fig. 1C and D). The NKX3.1 consensus sequence is similar, but not identical, to the binding sequences of other NK-2 class homeoproteins such as the CAAGTG site for Nkx2.1 (14) and TNAAGTG for Nkx2.5 (15). The consensus sequence for NKX3.1 is also similar to the TAAGTG consensus binding

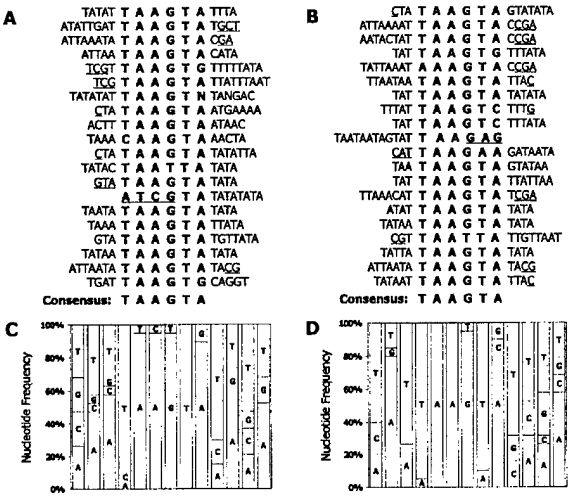

Figure 1. Determination of the NKX3.1 consensus DNA binding sequence. Sequences selected by (**A**) recombinant wild-type or (**B**) R52C NKX3.1 following five rounds of SAAB are listed. Underlined nucleotides represent non-random sequence. Nucleotides shown in bold represent those used in determination of the consensus binding sequence. The overall consensus binding sequence for each protein is listed below the aligned sequences. The percentage frequencies of each nucleotide for sequences selected by (**C**) wild-type NKX3.1 and (**D**) R52C NKX3.1 are shown.

sites of human Hox11 (22) and salmon Isl-2 (23), members of the Hox and LIM families of homeoproteins, respectively. The selected sequences also contained the TAAT motif recognized by a majority of homeodomain proteins with a characterized DNA-binding site (1). The TAAT motif was present at least once in 88% of the combined wild-type and R52C selected sequences. In similar binding assays Nkx2.5 and Isl-2 also selected sequences containing TAAT, in addition to their consensus binding sites (15,23).

Specific binding of wild-type and R52C NKX3.1 to the TAAGTA site was demonstrated by gel mobility shift (Fig. 2). Maltose-binding protein lacking the NKX3.1 moiety did not bind the TAAGTA sequence, suggesting that protein–DNA interactions were specifically due to the NKX3.1 segment of the fusion protein. Binding specificity was confirmed by competition with a 5-fold molar excess of unlabeled NKX3.1 consensus sequence, which competed for the TAAGTA probe. An Oct POU-1 homeodomain binding site was included as a control for non-specific DNA binding. The Oct site contains a CTAAAC binding site rather than the preferentially bound TAAGTA sequence. Neither protein bound the Oct probe.

Comparison of wild-type NKX3.1 binding to the NKX3.1, Nkx2.1 and Msx1 binding sites

The results of the SAAB assay indicated that NKX3.1 preferentially bound a TAAGTA sequence. Using a competitive binding assay, we compared wild-type NKX3.1 binding to the TAAGTA site with binding to the Nkx2.1 and Msx1 DNA binding sites. The Nkx2.1 sequence (14) has a CAAG core

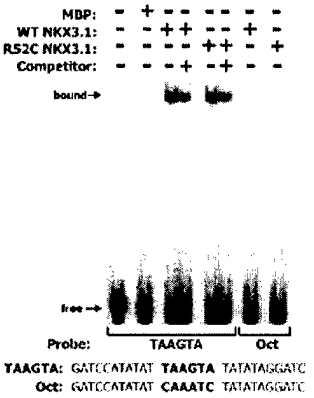

Figure 2. NKX3.1 specifically binds to the TAAGTA consensus binding site. Binding of maltose-binding protein, wild-type NKX3.1 and R52C NKX3.1 to a radiolabeled probe containing either the NKX3.1 consensus sequence (TAAGTA) or an Oct-1 binding site (CTAAAC) was analyzed by gel mobility shift. Free probe and protein-bound complex are indicated with arrows. Where indicated, a 5-fold molar excess of unlabeled TAAGTA sequence was included as competitor. Nucleotide sequences of the probes and competitor are listed at the bottom of the figure.

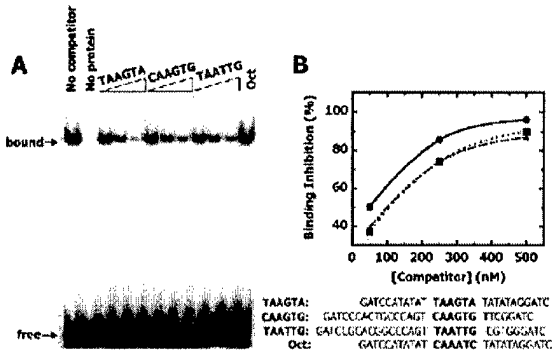

Figure 3. Wild-type NKX3.1 preferentially binds the NKX3.1 consensus sequence rather than Nkx2.1 and Msx1 binding sites. (**A**) Binding affinity of NKX3.1 to TAAGTA was compared with NKX3.1 binding to the Nkx2.1 and Msx1 binding sites by a competitive gel shift assay. Wild-type NKX3.1 was incubated with a probe containing the NKX3.1 consensus sequence in the absence or presence of unlabeled competitor DNA. Competitors were included at concentrations of 50, 250 and 500 nM. Free probe and protein-bound complex are indicated with arrows. (**B**) The data were quantitated and normalized to wild-type NKX3.1 binding to probe with no competitor. Nucleotide sequences of the competitors are listed at the bottom of the graph. closed circles, TAAGTA; closed squares, CAAGTG; closed triangles, TAATTG.

binding site, while the Msx1-binding site (24) contains the TAAT core sequence recognized by most homeoproteins. The nucleotide sequences of the Nkx2.1 (CAAGTG) and the Msx1 (TAATTG) binding sites are similar to the NKX3.1 consensus sequence, but differ at nucleotides proposed to be important for optimal binding (1,25). The results, shown in Figure 3A, suggest that wild-type NKX3.1 will bind to the Nkx2.1 and Msx1 consensus sites. These results are in agreement with a previous report that showed that a murine Nkx3.1 homeodomain polypeptide bound both CAAGTG and TAATTG sequences (5). However, the present data indicate that the NKX3.1 TAAGTA consensus site was reproducibly a slightly stronger competitor for NKX3.1 binding than either the CAAGTG or TAATTG binding sites (Fig. 3B). Using increasing amounts of protein with a constant probe concentration, Chen *et al.* also observed only minor differences in the binding affinity of Nkx2.5 when the CAAGTG NK-2 consensus sequence was substituted with either TAATTA or TAAGTG (15). As a control for non-specific competitor binding, an Oct-1 CTAAAC binding site, which was not bound by NKX3.1 (Fig. 1), was included as a competitor at 500 nM, the highest concentration used in the binding assay. There was no apparent decrease in the band representing protein-bound DNA in the presence of CTAAAC.

Effect of mutations in the TAAGTA consensus site on NKX3.1 binding

NKX3.1 belongs to the NK class of homeoproteins, but preferentially bound the novel TAAGTA site rather than the Nkx2.1 CAAGTG site. Competitive gel shift experiments were used to isolate the nucleotide(s) important for preferential binding of NKX3.1 to the TAAGTA site. Sequences were generated to replace either T with C at position 1 (TAAGTA→CAAGTA)

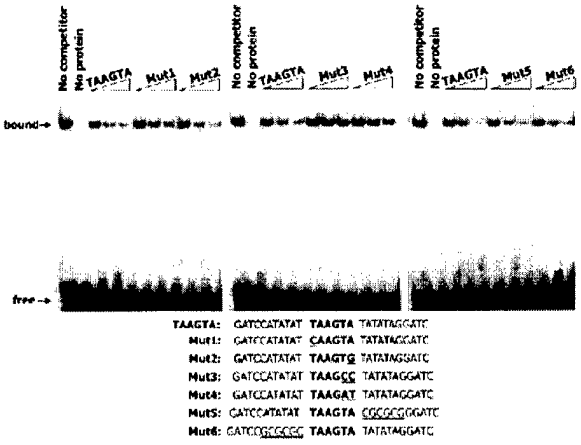

Figure 4. Effect of mutations in the TAAGTA consensus site on NKX3.1 binding. The effects of mutations in the NKX3.1 consensus site were analyzed by competitive gel shift assay. Wild-type NKX3.1 was incubated with a probe containing the NKX3.1 consensus sequence in the absence or presence of unlabeled competitor DNA. Competitors were included at concentrations of 50, 250 and 500 nM. Free probe and protein-bound complex are indicated with arrows. Nucleotide sequences of the competitors are listed at the bottom of the figure.

or A with G at position 6 (TAAGTA→TAAGTG) of the NKX3.1 consensus binding site. The results of the competition assay (Fig. 4) indicate that replacing A at position 6 of the consensus site with G did not affect binding competition relative to the native TAAGTA site. However, replacing T with C at position 1 decreased binding competition, suggesting

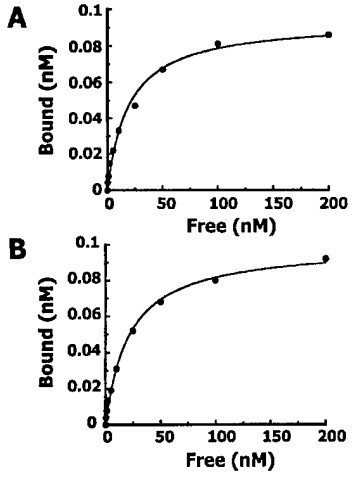

Figure 5. Wild-type and R52C NKX3.1 exhibit similar DNA binding affinities. The equilibrium dissociation constants for (**A**) wild-type and (**B**) R52C NKX3.1 binding to an NKX3.1 consensus site were determined by gel mobility shift using a constant amount of radiolabeled probe (1×10^{-10} M) with various protein concentrations ($0.5–200 \times 10^{-9}$ M). A plot of the quantitated data is shown as bound protein–DNA complex concentration as a function of free protein concentration. The data were analyzed by non-linear least squares as described in Materials and Methods.

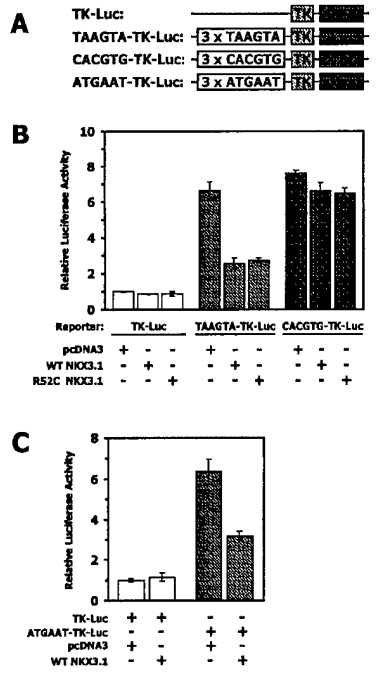

Figure 6. NKX3.1 represses reporter gene transcription. (**A**) Diagram representing reporter plasmids used to analyze the effect of NKX3.1 on reporter gene transcription. (**B**) Reporter plasmids were used to transiently transfect TSU-Pr1 cells co-transfected with wild-type or R52C NKX3.1 expression plasmids or empty expression plasmid (pcDNA3) as described in Materials and Methods. CMV-*Renilla* plasmid was included as a control for transfection efficiency. (**C**) TSU-Pr1 cells were co-transfected with expression plasmid and empty reporter vector or reporter vector containing NKX3.1 binding sites in the antisense direction. CMV-*Renilla* plasmid was included as a control for transfection efficiency.

that T instead of C at the first position of the consensus sequence is preferred for optimal NKX3.1 binding. Mutating the TAAGTA site to either TAAGCC (Mut3) or TAAGAT (Mut4) significantly decreased competitor binding (Fig. 4), indicating that the 3′-dinucleotide of the NKX3.1 consensus site is important for protein binding. These results are in agreement with previous findings by Damante *et al.* (25,26) indicating that the 3′-dinucleotide of the Nkx2.1 site (CAAGTG) is necessary for optimal protein binding. Sequences selected by wild-type and R52C NKX3.1 in the SAAB assay (Fig. 1) exhibited a high frequency of A/T-rich nucleotides flanking the TAAGTA binding site. To investigate the importance of the A/T flanking sequence for wild-type NKX3.1 binding, a competitive gel shift assay was performed using NKX3.1 binding sites with A/T flanking nucleotides replaced with G/C either upstream (Mut5) or downstream (Mut6) of the TAAGTA sequence (Fig. 4). Both Mut5 and Mut6 had similar competitor efficacy as the native NKX3.1 binding sequence, indicating that the predominance of A/T nucleotides flanking the TAAGTA site did not significantly influence NKX3.1 DNA binding. It is possible that the high frequency of A/T base pairs flanking the NKX3.1 consensus site was an artifact of the binding assay. In fact, previous selection assays using NK class homeoproteins also yielded sequences with a relatively high A/T content outside their respective consensus binding sites (15,17).

Wild-type and R52C NKX3.1 exhibit similar binding affinities

To determine if the polymorphism at position 52 affected DNA binding affinity, the dissociation constants for wild-type and R52C NKX3.1 binding to the TAAGTA consensus site were measured. Increasing amounts of protein were incubated with a constant amount of radiolabeled probe. Following quantitative analysis, the data were plotted and analyzed by non-linear least squares for dissociation constant determination (Fig. 5). The dissociation constants for the proteins were nearly identical, exhibiting K_d values of 20 nM for wild-type and 22 nM for R52C NKX3.1. The results indicate that mutation at position 52 of NKX3.1, which lies N-terminally outside the homeodomain region, does not affect DNA binding affinity.

NKX3.1 acts as a transcriptional repressor

Luciferase reporter vectors (Fig. 6A) were constructed with three tandem NKX3.1-binding sites in the sense (TAAGTA–TK–Luc) and antisense (ATGAAT–TK–Luc) directions upstream from a herpes simplex virus TK promoter. Co-transfection of TSU-Pr1 cells with TAAGTA–TK–Luc and an NKX3.1 expression vector resulted in decreased transcription compared with control cells co-transfected with TAAGTA–TK–Luc and empty expression vector (Fig. 6B), suggesting that in this context NKX3.1 behaved as a transcriptional

repressor. There was no apparent difference in the relative luciferase activities of wild-type and R52C NKX3.1 co-transfected with TAAGTA–TK–Luc, suggesting that the polymorphism did not affect NKX3.1 transcriptional repression *in vitro*. Previous binding studies have shown that a murine Nkx3.1 homeodomain will not bind to a CACGTG sequence (5). When the NKX3.1-binding sites were mutated to CACGTG, the relative luciferase activity using a NKX3.1 expression plasmid had little effect on transcription, indicating that transcriptional repression with reporter constructs containing three copies of TAAGTA was due to specific binding of NKX3.1. The level of transcriptional repression using ATGAAT–TK–Luc and wild-type NKX3.1 expression vector was similar to that observed using TAAGTA–TK–Luc and wild-type expression vector (Fig. 6C), indicating that the orientation of the TAAGTA sequence does not affect transcriptional repression due to NKX3.1 binding. Insertion of the TAAGTA and CACGTG inserts increased luciferase activity relative to the unmodified TK–Luc reporter plasmid. Based on these results, it is likely that these inserts contain binding sites for transcriptional activators endogenously expressed in TSU-Pr1 cells. For example, the TAAGTA insert contains multiple ATTA sequences (the complement of TAAT), which is bound by most homeoproteins (1). Also, the CACGTG sequence is a high affinity MYC-binding site (27).

DISCUSSION

Using purified fusion proteins in a random DNA selection assay, a TAAGTA consensus binding sequence for wild-type and R52C NKX3.1 was identified. The TAAGTA consensus site has not been isolated for any other NK class homeoprotein. Previously reported DNA binding sites for NK class homeoproteins include CAAGTG for Nkx2.1, AAAGTG for *Caenorhabditis elegans* ceh-22 and NAAGTG for Nkx2.5 (14,15,28). Structural and mutational studies indicate that amino acid residues of helix III and the N-terminal arm of the homeodomain primarily determine DNA binding specificity (26). Amino acid residues 6–8 of the N-terminal arms of the NK-2 and Nkx2.1 homeodomains have been implicated as the primary determinants of the preferred nucleotide for binding at the 5′-end of the 5′-CAAG-3′ core binding site (26,29). The homeodomains of NK-2 and Nkx2.1 have Val, Leu and Phe at positions 6–8, while NKX3.1 has Ala, Ala and Phe at these positions. The variation in side-chain volume of residues 6 and 7 may result in preferential binding by NKX3.1 of T instead of C at the first position of the TAAGTA binding site. When Leu at position 7 of the NK-2 homeodomain was mutated to Ala, the binding affinity for the CAAGTG site decreased by one order of magnitude (29), further suggesting that side-chain volume of residues 6 and 7 is an important determinant of homeodomain binding specificity. Attempts to alter binding preference from TAAGTA to CAAGTA by mutation of Ala to Leu at position 7 of the NKX3.1 homeodomain were unsuccessful (our unpublished data). Therefore, it is likely that a relatively small side-chain volume at position 6, in addition to position 7, of the NKX3.1 homeodomain is required for preferential binding of T instead of C in the TAAGTA sequence. NK class homeodomains characteristically have Tyr at position 54 (2), which confers preference for atypical DNA binding sites relative to the canonical TAAT binding site recognized by most homeodomains (26). The tyrosine residue at position 54 of the homeodomain is reportedly involved in the recognition and preferential binding of guanosine at the 3′-end of the 5′-CAAG-3′ core binding site for Nkx2.1 (26). NKX3.1 also has Tyr at position 54 of the homeodomain, therefore, it is not surprising that wild-type NKX3.1 preferentially binds a TAAG rather than a TAAT core sequence (Fig. 3). Optimal DNA binding of Nkx2.1 requires a TG dinucleotide following the CAAG core binding site (25). The consensus DNA binding sequence for NKX3.1 exhibited a TA dinucleotide following the TAAG core. Structural studies of Nkx2.1 have revealed that Gln at position 50 of the homeodomain region contacts the TG dinucleotide flanking the 3′-end of the 5′-CAAG-3′ core binding sequence. This residue is conserved among NK-2 and NK-3 homeodomains (2). Therefore, based on sequence homology, it is predicted that NKX3.1 would also preferentially bind a TG dinucleotide instead of TA following the TAAG core binding site. Although our binding site selection data suggest that NKX3.1 prefers a TA dinucleotide following the TAAG core (Fig. 4), competitive binding experiments suggested that replacing the A with G in the dinucleotide (TAAGTA→TAAGTG) had no observable effect on wild-type NKX3.1 binding (Fig. 4), providing evidence that the nucleotides may be interchangeable. In fact, a previous report describing the DNA sequences within the thyroglobulin promoter important for Nkx2.1 transactivation identified a CAAGTA binding site as well as CAAGTG (30).

Based on the results of competition gel shift assays, NKX3.1 displays a high degree of binding promiscuity (Fig. 3). It is likely that site-specific DNA binding of NKX3.1 *in vivo* is enhanced by post-translational modification and/or protein–protein interaction. For example, phosphorylation and intermolecular disulfide bond formation have been shown to increase DNA binding affinities of Nkx2.1 and Nkx2.5 (31–33). Also, protein interaction of NK-3 with the homeodomain-interacting protein kinase 2 (HIPK2) significantly increased NK-3 DNA binding affinity and transcriptional repression, independently of NK-3 phosphorylation by HIPK2 (34). In addition, Choi *et al.* have recently shown that the co-repressor protein Groucho, which by itself does not repress transcription, interacts with the homeodomain region of NK-3 to increase *in vitro* transcriptional repression (35).

There were no apparent differences in DNA binding or transcriptional repression between wild-type and R52C NKX3.1 under the described conditions. The polymorphism occurs at position 52 of NKX3.1, which is not located within the homeodomain. It has been suggested that regions outside the homeodomain may not contribute to DNA binding (25). However, it is possible that wild-type and R52C NKX3.1 differ functionally *in vivo* due to post-translational modification(s). For example, our preliminary data suggest that the polymorphism at position 52, which lies within a putative phosphorylation site at Ser48 (unpublished observation), may affect DNA binding through altered levels of phosphorylation (manuscript in preparation). Also, introduction of a cysteine at position 52 may affect redox sensitivity of DNA binding by the formation of aberrant inter- or intramolecular disulfide bonds. Further analysis may provide insights into the effects of the polymorphism on the function of NKX3.1.

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A Common Polymorphism of the Prostate-Specific Homeoprotein NKX3.1 Affects Phosphorylation and DNA Binding

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Abstract

NKX3.1 is a member of the NK-class of homeodomain proteins and is expressed primarily in the adult prostate. Previously, a C→T polymorphism at nucleotide 154 of *NKX3.1* was identified, resulting in substitution of Arg with Cys at amino acid 52 of NKX3.1 (R52C). The polymorphism is found in 8% of a cohort of African American men and in 17% of a cohort of Caucasians. Amino acid 52 lies adjacent to a consensus phosphorylation site at serine 48. The polymorphic protein had reduced phosphorylation *in vivo* compared to the wild-type protein. *In vitro* phosphorylation of NKX3.1 (R52C) was reduced by more than 70% compared to wild-type protein. In cells, NKX3.1 is phosphorylated only on serine residues. Phosphorylation of wild-type NKX3.1 at serine 48 resulted in diminished DNA binding affinity in vitro. In contrast, phosphorylation of NKX3.1 R52C did not affect DNA binding, suggesting that the polymorphism caused loss of a mechanism of controlling NKX3.1 activity. Consistent with this observation, NKX3.1 R52C conferred greater transcriptional repression on an MMTV-LTR reporter construct than did wild type NKX3.1.

Introduction

NKX3.1 is an androgen-regulated NK-class homeobox gene (1-3) with expression in the adult localized primarily in the prostate (2, 3). *NKX3.1* has been mapped to chromosome 8p21 (2, 4), a locus frequently deleted in prostate cancer (5-7). However, no cancer-specific mutations of the NKX3.1 coding region have been identified by genetic analysis of human prostate cancer samples (4). The murine Nkx3.1 protein homolog is approximately 70% conserved compared to the human protein. Targeted disruption of murine *Nkx3.1* suggested that the gene exerts growth suppression and differentiating effects on prostatic epithelium (8, 9). Immunohistochemical analysis of human prostate cancer tissues revealed loss of NKX3.1 expression was associated with hormone refractory disease and advanced tumor stage (Bowen et al., in press).

In the course of analyzing tumor samples for *NKX3.1* mutations we found a C→T polymorphism at nucleotide 154 that resulted in substitution of Cys for Arg at amino acid 52 of NKX3.1 (4). The polymorphism lay N-terminal to the homeodomain in a region of the protein that was not conserved between mouse and human. Analysis of the consensus DNA-binding site for NKX3.1 showed that recombinant wild-type and mutant ($R52C^1$) NKX3.1 both identified the sequence TAAGTA with similar binding affinities (10). Both wild-type and R52C NKX3.1 had similar effects on a transcription reporter vector with multiple upstream NKX3.1 consensus binding sites upstream from a thymidine kinase promoter (10).

Homeoproteins are known to undergo posttranslational modification by phosphorylation. Homeoprotein phosphorylation has been shown to affect protein-protein interactions (11), subcellular localization (12), DNA binding affinity (13), and

transcriptional activity (14). Generally, these effects have been attributed to electrostatic repulsion or a conformational change in the protein (15). Members of the NK-family of homeoproteins have been shown to undergo phosphorylation. The kinases responsible for phosphorylating NK-class homeoproteins include casein kinase II (CKII) (14), MST2 kinase (16), extracellular signal-regulated kinase (17), homeodomain interacting protein kinase (18), protein kinase A (PKA) (19), and protein kinase C (PKC) (20). We utilized a database to analyze the NKX3.1 amino acid sequence for consensus phosphorylation sites. Several consensus phosphorylation sites for a variety of protein kinases were identified. In particular, the database search identified Ser48 of NKX3.1 as a consensus phosphorylation site for Calmodulin Kinase II (CaMII), PKA and PKC. Because of its proximity to the R52C polymorphism, we characterized NKX3.1 phosphorylation at this site and showed that it affected DNA binding and transcriptional repression. Moreover, R52C affected the degree of phosporylation at serine 48 *in vitro* and *in vivo*.

MATERIALS AND METHODS

Plasmid Construction. Plasmids expressing full-length wild-type or polymorphic NKX3.1 fused to maltose-binding protein were generated as previously described (10). Plasmid encoding amino acids 1-184 (nucleotides 1-581) of wild-type NKX3.1 was prepared as follows. NKX3.1 cDNA with an N-terminal FLAG epitope was created by PCR amplification using the upstream primer 5'-CGGGATCCGAATTCATGGATTACAAGGATGACGACGATAAGCTCAGGGTTCC GGAGCCGC-3' and the downstream primer 5'-GGGCCTCGAGTCTAGATGAGCTGCTTTCGCTTAGTCTTATAGC-3'. The upstream primer contains an EcoRI site and the downstream primer has an XhoI

restriction site (restriction sites underlined) for cloning. Wild-type NKX3.1 cDNA was used as template and the amplified DNA was cloned into pCRII-Topo using a Topo-TA cloning kit (Invitrogen, Carlsbad, CA). Following digestion of the resulting vector with EcoRI and XhoI, the fragment was isolated and ligated into pcDNA3.1 (Invitrogen). The plasmid was sequenced at the 5'-end of the insert to ensure reading frame fidelity. NKX3.1 point mutants were generated using a Quikchange Site-Directed Mutagenesis Kit (Strategene, La Jolla, CA) and following the manufacturer's protocol. Mutant NKX3.1 cDNAs were fully sequenced to confirm the presence of mutation and to ensure that no additional mutations were introduced. A reporter vector containing the mouse mammary tumor virus long terminal repeat (MMTV-LTR) promoter in plasmid pCAT5 was obtained from Dorraya El-Ashry, Lombardi Cancer Center. The MMTV-LTR was cloned into the pGL3-Basic vector (Promega, Madison WI) upstream of the luciferase reporter gene.

Bacterial Expression and Purification of NKX3.1 Fusion Proteins. Plasmids expressing wild-type or R52C NKX3.1 were used to transform competent *Escherichia coli* strain BL21. The proteins were expressed and purified as previously described (10). Briefly, transformants were used to inoculate 10 mL Luria Bertani (LB) medium and the cultures were incubated with shaking overnight. The 10 mL culture was used to inoculate 1 L of LB medium containing 0.2 % glucose and incubated with shaking until the optical density at 600 nm reached 0.6. IPTG was added to a final concentration of 0.3 mM and the culture was incubated for an additional 60 min. Cells were harvested and resuspended in 10 mL of buffer A (20 mM Tris-HCl, 200 mM NaCl, 1 mM EDTA, and 1 mM dithiothreitol). Suspended cells were lysed by sonication and a cell-free extract was

prepared by centrifugation. The cell-free extract was loaded onto an amylose column (New England Biolabs, Beverly, MA) and washed with buffer A. NKX3.1 fusion proteins were eluted from the column with buffer A including 10 mM maltose.

In Vivo Phosphorylation and Immunoprecipitation. For labeling of exogenous NKX3.1, TSU-Pr1 or LNCaP cells were plated on a 6 cm dish in DMEM containing 5% fetal bovine serum (FBS; Gibco BRL, Rockville, MD). At approximately 90% confluence, cells were transfected with 10 μg of wild-type or mutant NKX3.1 or empty expression vector using Lipofectamine 2000 following the manufacturer's protocol (Gibco). Approximately 48 hr post-transfection, growth medium was replaced with phosphate-free DMEM containing 5 % dialyzed FBS (Gibco). Cells were treated with 1 mCi/ml [^{32}P]orthophosphate in carrier free HCl (Amersham Pharmacia Biotech, Piscataway, NJ) for 3-4 hr. For labeling of endogenous NKX3.1, LNCaP cells were plated on a 6 cm dish and incubated until cells were approximately 85% confluent. Medium was changed to serum-free medium (Gibco) containing 5% charcoal stripped serum (Gibco). The synthetic androgen R1881 (Dupont, Boston, MA) was then added to a final concentration of 1 nM to induce the expression of NKX3.1. 48 hr following addition of R1881, the cells were treated with [^{32}P]orthophosphate for 4 hr. Cells were then treated with or without 100 nM TPA (Sigma, St. Louis, MI) for 30 min prior to cell lysis. Labeled TSU-Pr1 and LNCaP cells were lysed in 0.5 ml RIPA buffer (50 mM Tris, pH 7.2, 150 mM NaCl, 1% Triton X-100, 0.1 % SDS, and 1% sodium deoxycholate, 50 mM NaF, and 10 mM Na_2HPO_4, pH 7.2) and cell debris was removed by centrifugation (20,000 × g at 4 °C). Supernatant was transferred to a new tube and 1.5 μg of anti-NKX3.1 polyclonal antiserum (Bowen et al., in press) or 20 μg of anti-FLAG M2 antibody (Stratagene) was

added for incubation at 4 °C for 1 hr. Following incubation, 50 µl of Protein G plus Protein A agarose (Calbiochem, San Diego, CA) were added to the sample and rotated at 4 °C for 1 hr. Beads were washed 3 times with 0.5 ml RIPA buffer followed by addition of 5 µl of 6X SDS-PAGE sample buffer. Immunoprecipitants were electrophoresed on denaturing 10-20% gradient polyacrylamide gels followed by gel drying and autoradiography for visualization of radiolabeled proteins.

Phosphoamino Acid Analysis of NKX3.1. Labeled proteins were excised and eluted from polyacrylamide gels. The eluted protein was digested with 0.15 mg/ml trypsin overnight at 37°C followed by hydrolysis with 1 ml of 6 N HCl at 105 °C for 1 hr. HCl was removed by lyophilization and pellet was washed with 1 ml H_2O and dried. The pellet was resuspended in H_2O and phosphoamino acids were separated by one dimensional thin-layer electrophoresis as described (21). The identity of *in vivo* phosphorylated amino acids was determined by autoradiography followed by comparison of the autoradiogram with phosphoamino acid standards.

In Vitro Phosphorylation. Synthetic peptides (30 µg) obtained from Research Genetics, Inc. (Huntsville, AL) or purified fusion proteins (200 ng) were incubated with 10 ng of a purified PKC α, β and γ isoform mixture (Upstate Biotechnology, Lake Placid, NY) in a buffer containing 20 mM HEPES, pH 7.4, 10 mM $MgCl_2$, 0.2 mM EGTA, 5 mM DTT, 0.5 mM $CaCl_2$, 100 µg/mL phosphatidylserine, 5 mM DTT, 50 µM ATP, and 0.11 µCi [γ-^{32}P]ATP. Following incubation at 30°C for 30 min, the phosphorylated proteins or peptides were either separated on an 8% denaturing polyacrylamide gel followed by autoradiography or transferred to a P81 disc (Whatmann, Clifton, NJ) and radioactivity determined by liquid scintillation counting. Non-radioactive phosphorylation of the

fusion proteins for gel shift assays was performed as described above without the addition of [γ-^{32}P]ATP. Following non-radioactive phosphorylation, an equal volume of glycerol was added to the protein solution for storage at –20°C until needed.

Western Blot Analysis. Western blotting was performed as described previously (Bowen et al., in press). Purified proteins were electrophoresed on a 10-20% denaturing gradient polyacrylamide gel. Proteins were transferred to a nitrocellulose membrane (Bio Rad, Hercules, CA) in Transfer buffer (12 mM Tris, 96 mM glycine, 20% methanol) for 1 hr at 25 V at room temperature. Blots were washed 1 hr in phosphate buffered saline with 0.1% Tween-20 (PBS-T). Blots were incubated overnight at 4°C in PBS-T supplemented with 5% milk. Blots were washed with PBS-T and incubated with either an anti-maltose binding protein rabbit antiserum (New England Biolabs, Beverly, MA), an affinity purified anti-NKX3.1 antiserum (Bowen et al., in press) or an anti-FLAG M2 antibody (Stratagene). Blots were washed with in PBS-T and incubated with a secondary antibody conjugated with horse radish peroxidase. The blots were washed with PBS-T and exposed to Supersignal reagent (Pierce, Rockford, IL). Film was exposed to the blots for visualization of protein bands.

Electrophoretic Mobility Shift Assay. Gel shift assays were performed as described previously (10) with modification. Briefly, double-stranded DNA representing a consensus NKX3.1 binding site (10) was prepared by annealing equimolar amounts of complimentary oligonucleotides (upper strand, 5'-GTATATAAGTAGTTG-3'). The double-stranded DNA was radiolabeled with [γ-^{32}P]ATP using T4 polynucleotide kinase. The labeled probe (5 nM) was added to 2, 5, 10 or 25 ng purified NKX3.1 in binding buffer (10 mM Tris-Cl, pH 7.5, 50 mM NaCl, 7.5 mM $MgCl_2$, 1 mM EDTA, 5%

glycerol, 5% sucrose, 0.1% Nonidet P-40, 1.0 μg BSA, 0.1 μg polydIdC, and 5 mM DTT) to a final volume of 20 μL. Protein-DNA complexes were separated from unbound probe by native 8 % PAGE at 175 V for 1 hr at ambient temperature in 0.5× TBE buffer following pre-electrophoresis of the gel at 200 V for 60 min. Film was exposed to the dried gels and representative gel shift assays are shown.

Reporter Gene Assay. CV-1 cells were cultured in Improved Minimal Essential Medium (Gibco BRL) supplemented with 5% fetal calf serum. Cells were transiently transfected using 6×10^4 cells per well of a 24-well plate with 250 ng of reporter plasmid, 250 ng androgen receptor expression plasmid, 0 – 2.0 μg NKX3.1 expression plasmid and 1 ng of CMV *Renilla* plasmid in the presence of 2 μg of LipofectAMINE 2000 (Gibco BRL) following the manufacturer's protocol. The transfectants were treated with or without 1 nM dihydrotestosterone (Steraloids, Wilton, NH). Cells were lysed 48 hr after transfection and the lysate was assayed for firefly and *Renilla* luciferase activities using Dual Luciferase Reporter Assay reagents (Promega, Madison, WI). The data represent the average of 3 separate experiments normalized by *Renilla* luciferase activity.

TaqMAN Assay. The TaqMan Allelic Discrimination assay (22) was used to determine the frequency of the polymorphism at nucleotide 154 in prostate DNA samples. Genomic DNA was isolated using the Easy DNA Genomic DNA Isolation Kit (Invitrogen, Carslbad, CA). The probe used to detect the wild-type codon was: 5'-CAGAGACAGCGCGACCCGG-3' and the probe used to detect the polymorphic codon was: 5'-CAGAGACAGTGCGACCCGGAGC-3'. The wild-type probe contained a 5'-FAM (6-carboxyfluorescein) reporter dye, while the polymorphic probe had a 5'-TET (6-carboxy-4,7,2',7'-tetrachlorofluoroscein) reporter dye. Both probes had a 3'-TAMRA (6-

carboxy-N,N,N',N'-tetramethylrhodamine) quencher dye. Probes used for allelic discrimination were synthesized by Biosearch Technologies, Inc. (Novato, CA). The forward primer used for PCR was 5'-CGCAGCGGCAAGGC-3' and the reverse primer was 5'-GGTGCTCAGCTGGTCGTTCT-3' (GibcoBRL, Rockville, MD). Taqman Universal PCR Master Mix (Applied Biosystems, Foster City, CA) was used in the PCR reaction following the manufacturer's protocol. DNA (100 ng), primers (900 nM each) and probe (100 nM FAM-tagged or 200 nM TET-tagged) were added to the TaqMan Universal PCR Master Mix in a total volume of 50 μL. PCR was carried out on an an ABI Prism 7700 Sequence Detection System (Applied Biosystems) using the following program: 50 °C for 2 min, 95 °C for 10 min and 40 cycles of 95 °C for 15 sec and 62 °C for 1 min. Allelic discrimination analysis was performed on an ABI Prism 7700 Sequence Detection System (Applied Biosystems).

Results

NKX3.1 is Phosphorylated at Serine In Vivo. To determine if NKX3.1 is phosphorylated *in vivo*, TSU-Pr1 cells were transfected with a wild-type NKX3.1 expression construct or empty vector. The NKX3.1 construct contained cDNA for expression of amino acids 1-184 including the N-terminus and homeodomain of NKX3.1 (Fig. 1). Our unpublished data indicated that expression of transfected NKX3.1 was signinficantly enhanced in TSU-Pr1 and LNCaP cells by deletion of amino acids 185-234. Treatment of TSU-Pr1 cells transfected with the wild-type NKX3.1 construct using [^{32}P]orthophosphate followed by immunoprecipitation with an affinity purified anti-NKX3.1 antiserum detected phosphorylated protein that migrated at approximately 25 kDa (Fig. 2A.). Cells transfected with empty vector or cell lysates immunoprecipitated with pre-immune serum

did not yield the 25 kDa band when treated with [^{32}P]orthophosphate. This band corresponds to the predicted 23 kDa molecular weight of amino acids 1-184 of NKX3.1. As a control for antibody specificity, parallel cultures of TSU-Pr1 transient transfectants were prepared for western blotting. The antibody used for immunoprecipitation was used as primary antibody in the western analysis. No NKX3.1 protein was detected in lysates transfected with the empty vector. The protein detected in lysates transfected with NKX3.1 expression vector also migrated at approximately 25 kDa. NKX3.1 expression is upregulated in LNCaP cells treated with androgen (2, 23). Endogenously expressed NKX3.1 from LNCaP cells treated with R1881 was also phosphorylated *in vivo* and the level of phosphoprotein was increased by the presence of 100 nM TPA (Fig. 2B), suggesting that NKX3.1 is phosphorylated *in vivo* by a TPA-induced kinase such as PKC. Treatment of the cells with TPA did not cause an increase in the level of NKX3.1 protein as determined by Western blotting (Fig. 2B). Phosphoamino acid analysis of the radiolabeled endogenous protein indicated that NKX3.1 is phosphorylated only at serine (Fig. 2C). Similar phosphoamino acid analysis results were obtained with exogenously labeled NKX3.1 from TSU-Pr1 cells (data not shown).

Wild-Type and Mutant NKX3.1 are Differentially Phosphorylated by PKC. The software program Phosphobase v2.0 (24) was used to analyze the NKX3.1 amino acid sequence for possible phosphorylation sites. Of the consensus sites identified, Ser48 was a candidate phosphorylation site for CaMII, PKA and PKC. This site was of interest because it is located in close proximity to amino acid 52, the site of the NKX3.1 polymorphism. Previously, Zannini *et al.* showed that Nkx2.1 could be phoshphorylated by PKC (20). To determine if NKX3.1 could also be phosphorylated by PKC, purified

wild-type fusion protein was incubated with [γ-^{32}P]ATP in the presence of a purified mixture of the α, β and γ isoforms of PKC. The results shown in Fig. 3A demonstrate that wild-type NKX3.1 fusion protein was phosphorylated *in vitro* by PKC. In addition to NKX3.1 phosphorylation, a minor level of PKC autophosphorylation was present, represented by the 80 kDa band. Phosphobase v2.0 also identified four consensus PKC sites in the amino acid sequence of the maltose-binding protein affinity tag. However, PKC did not phosphorylate maltose-binding protein alone, suggesting that phosphorylation of the fusion protein was specific for the NKX3.1 moiety.

To determine if the polymorphism could affect phosphorylation of NKX3.1, R52C fusion protein was also used as a substrate in a PKC kinase assay. The results shown in Fig. 3A indicate that PKC phosphorylation of R52C NKX3.1 was noticeably decreased relative to phosphorylation of wild-type NKX3.1. To ensure that equal amounts of the proteins were loaded onto the gel, samples were prepared in parallel without [γ-^{32}P]ATP. The samples were electrophoresed and blotted with an anti-maltose binding protein antiserum. The results shown in the bottom panel of Fig. 3A indicate that equal amounts of fusion protein with either wild-type or R52C NKX3.1 were loaded onto the gel.

To verify that the mutation at position 52 negatively affected phosphorylation, synthetic peptide substrates representing amino acids 43-54 of NKX3.1 were used in PKC kinase assays. The results of the kinase assays using the peptide substrates shown in Fig. 3B were consistent with PKC phosphorylation of the full-length proteins. Relative phosphorylation of peptide P-WT representing wild-type NKX3.1 was 3-fold higher than peptide P-R52C representing R52C NKX3.1. Phosphorylation of peptide P-S48A with

Ser replaced by Ala at the relative position 48 of NKX3.1 was decreased 33-fold relative to peptide P-WT, indicating that phosphorylation was specific for Ser48. To determine if the effects of the R52C mutation *in vivo*, wild-type, R52C or S48A NKX3.1 mammalian expression vectors were used to transfect LNCaP cells. The R52C mutation decreased phosphorylation of NKX3.1 by 2-fold. Mutating Ser48 to Ala significantly decreased the level of *in vivo* phosphorylation of NKX3.1 (Fig. 3C), providing evidence that Ser48 is a phosphoacceptor.

Phosphorylation by PKC Represses Wild-Type but not R52C NKX3.1 DNA Binding. The effects of PKC phosphorylation on NKX3.1 DNA binding were determined by gel shift assays. Purified NKX3.1 fusion proteins were treated with PKC in the presence or absence of ATP and the proteins were included in gel shift assays with a radiolabeled NKX3.1 consensus DNA binding sequence (10). Phosphorylation of wild-type NKX3.1 decreased the apparent binding affinity of the protein for the consensus sequence by 3-fold relative to the nonphosphorylated protein (Fig. 4). However, the apparent DNA binding affinity of R52C was not noticeably altered upon treatment of PKC in the presence of ATP. To determine if Ser48 of NKX3.1 was directly involved in modulating DNA binding affinity due to PKC phosphorylation, fusion protein with Ser48 mutated to Ala (S48A) was included in a gel shift assay. Replacing Ser with Ala disrupted phosphorylation-regulated DNA binding (Fig. 4), indicating that the phosphorylation state of Ser48 modulates NKX3.1 DNA binding affinity. For controls, the two other consensus PKC phosphorylation sites at Thr119 and Thr179 were mutated to Ala. PKC treatment of NKX3.1 with T119A yielded results that were similar to wild-type NKX3.1

(Fig. 4). Interestingly, mutating Thr179, which is in the homeodomain, to Ala abolished NKX3.1 DNA binding, regardless of protein phosphorylation (Fig. 4).

R52C Polymorphism Affects Androgen-Regulated Reporter Gene Expression. The MMTV-LTR has an androgen response element (ARE) that mediates transcriptional activation by the androgen receptor (25, 26). The ARE is located 449 nucleotides from the 3'-end of the LTR and 151 nucleotides upstream of the TATA box. Expression of the reporter construct was activated by the addition of an AR expression plasmid in the presence of 1 nM DHT (Fig. 5). Cotransfection of wild-type NKX3.1 or NKX3.1 R52C expression plasmid with the reporter vector in the presence of DHT decreased reporter gene expression in proportion to the amount of NKX3.1 plasmid added. These results are consistent with our previous data indicating that NKX3.1 could repress transcription of luciferase from a reporter vector with a thymidine kinase promoter with tandem upstream TAAGTA binding sites (10). Co-transfection of NKX3.1 R52C expression plasmid enhanced transcriptional repression of the luciferase reporter gene by 115–120 % relative to wild-type NKX3.1 (Fig. 5). These results are in agreement with the idea that the R52C polymorphism decreases NKX3.1 phosphorylation, resulting in deregulation of NKX3.1 DNA binding affinity.

Prevalence of R52C Polymorphism. To determine the frequency that the NKX3.1 R52C polymorphism occurred in the population and to carry out a preliminary investigation of whether NKX3.1 R52C affected risk for prostate cancer, we analyzed germ line DNA from age-matched Caucasian and African American cases and controls in a cohort study of prostate cancer. Overall, 13% of men in the study were found to carry the NKX3.1 R52C polymorphism. There was a somewhat higher prevalence of the polymorphism

among Caucasians than African Americans. In this small sample there was no indication that cancer risk was conferred by NKX3.1 R52C (Table 1).

Discussion

Using *in vivo* labeling techniques, we were able to determine that NKX3.1 is phosphorylated *in vivo* only at serine residues (Fig. 2). At present, this is the first reported analysis of NKX3.1 phosphorylation. Mutating Ser48 to Ala reduced the level of NKX3.1 phosphorylation in LNCaP cells (Fig. 3C), indicating that Ser48 is a target residue for *in vivo* phosphorylation. However, the S48A mutation did not completely abolish *in vivo* phosphorylation, suggesting that NKX3.1 is phosphorylated at one or more other serine residues. Kasahara and Izumo recently reported CKII phosphorylation of Ser163 of the NK-class homeoprotein Nkx2.5 resulting in an increased target DNA binding affinity (14). This residue is highly conserved among NK-class homeoproteins (14) and is present at the equivalent location in the homeodomain of NKX3.1 at position 150 (2). It is possible that Ser150 of NKX3.1 is also phosphorylated by CKII in the NKX3.1 homeodomain, thereby altering NKX3.1 DNA binding affinity.

Wild-type and R52C NKX3.1 could be phosphorylated *in vivo* and *in vitro* (Fig. 2 and 3). However, wild-type NKX3.1 is preferentially phosphorylated relative to R52C NKX3.1 (Fig. 3). The substrate and site specificity of protein kinases have been shown to depend largely on the primary amino acid sequence surrounding the target residue of phosphorylation (27-29). For example, synthetic peptide substrates have been used to analyze the substrate specificity of PKC and researchers have demonstrated that additional basic residues located N- and C-terminally to the target amino acid enhance PKC catalytic rate and substrate specificity (28, 30, 31). Based on these results, it is

likely that decreased PKC phosphorylation of R52C relative to wild-type NKX3.1 results from reduced turnover and specificity of the polymorphic protein due to replacement of a basic residue (Arg) with a neutral residue (Cys) at position 52.

Although Ser48 of NKX3.1 lies N-terminally outside of the DNA binding region, the results suggest that phosphorylation of this residue is directly involved in the regulation of DNA binding (Fig. 4). Previous reports have described altered DNA binding of *Drosophila* Engrailed and Antennapedia homeoproteins due to phosphorylation of target amino acids located outside of their respective DNA binding regions (13, 32). Altered DNA binding due to phosphorylation of residues located outside of a protein's DNA binding domain has been proposed to be due to a structural change that occurs upon phosphorylation, resulting in a conformation that upregulates or downregulates protein-DNA interaction (13).

Interestingly, mutating Thr at position 179 of NKX3.1 (amino acid 56 of the homeodomain) completely abolished DNA binding (Fig. 4), indicating that this residue is critical for NKX3.1 DNA interaction. In fact, Thr is present at position 56 of all NK-3 class homeodomains (1, 2, 33-35). It is unlikely that replacing Thr179 with Ala disrupts DNA binding due to a change in volume since the side chain volumes of Thr and Ala are similar (36). Therefore, it is likely that the hydroxyl group of Thr at position 179 of NKX3.1 is involved in a structurally critical hydrogen bonding network. *Drosophila* NK-2 also has Thr at position 56 of the homeodomain, however this residue is not conserved among NK-2 class family members (37). Replacing Thr56 of the NK-2 homeodomain with Trp had no effect on DNA binding relative to the wild-type (37), indicating that

Thr56 may not be critical for all NK-class homeoproteins with Thr at this relative position.

We have described the DNA binding region of NKX3.1 and constructed a reporter that had 3 tandem NKX3.1 binding sites upstream of a TK promoter (10). Wild-type and R52C NKX3.1 equally repressed transcription of this reporter vector (10). Here we have shown that repression of transcription from the MMTV-LTR promoter by NKX3.1 R52C was stronger than by wild-type NKX3.1. Therefore, action of NKX3.1 and the polymorphic variant may be promoter-dependent. Another explanation lies in the possibility that NKX3.1 interacts with the AR resulting in decreased androgen-regulated transcriptional activation. A recent report by Kasahara *et al.* described an R→C polymorphism at amino acid 25 of NKX2.5, which lies N-terminal to the homeodomain (38). The polymorphism did not significantly affect DNA binding affinity or transcriptional activity relative to the wild-type protein. However, the polymorphism downregulated protein-protein homodimer formation of NKX2.5. Therefore, the R52C polymorphism could affect homodimerization of NKX3.1 or even affect a putative NKX3.1-AR interaction. Identification of physiologic promoter targets of NKX3.1 will provide further insight into the activity of wild type and polymorphic proteins.

Footnotes

[1]The abbreviations used are: R52C, arginine replaced by cysteine at position 52 of NKX3.1; S48A, serine replaced with alanine at amino acid 48 of NKX3.1; T119A, threonine replaced with alanine at position 119 of NKX3.1; T179A, threonine replaced with alanine at position 179 of NKX3.1; CKII, Casein Kinase II; PKC, Protein Kinase C;

PKA, Protein Kinase A; CaMII, Calmodulin Kinase II; PCR, polymerase chain reaction; IPTG, isopropyl-β-D-thiogalactopyranoside; R1881, methyltrienolone; ATP, adenosine-5'-triphosphate; SDS-PAGE, sodium dodecyl polyacrylamide gel electrophoresis; DMEM, Dulbecco's Modified Eagles Medium; FBS, fetal bovine serum; AR, androgen receptor.

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Figure Legends

Figure 1. Schematic diagram of NKX3.1. The numbering of amino acids is shown above the diagram. Consensus PKC phosphorylation sites are shown below the diagram. Target amino acids at Ser48, Thr119 and Thr179 are underlined. For reference, the position of the polymorphism at position 52 of NKX3.1 is shown.

Figure 2. NKX3.1 is phosphorylated *in vivo*. A, TSU-Pr1 cells were transfected with a wild-type NKX3.1 expression vector or empty vector. 48 hr post-transfection, cells were treated with 1 mCi/ml [^{32}P]orthophosphate. Cells were then lysed and NKX3.1 was immunoprecipitated with an anti-NKX3.1 antiserum or mouse IgG. Immunoprecipitants were electrophoresed by SDS-PAGE and phosphorylated protein was visualized by autoradiography. The same antibody used for immunoprecipitation of NKX3.1 was used in a Western blot of lysates from cells transfected with either empty vector or NKX3.1 expression vector. B, LNCaP cells were treated with R1881 and 48 hr later the cells were

exposed to 1 mCi/ml [^{32}P]orthophosphate. Cells were then treated with or without TPA (100 nM) for an additional 30 min. Cells were lysed and NKX3.1 was immunoprecipitated with an anti-NKX3.1 antibody, electrophoresed and radiolabeled proteins were visualized by autoradiography (top). Western blotting using an anti-NKX3.1 antibody (1.5 μg) was used to control for protein loading (bottom). C, Endogenous radiolabeled NKX3.1 was excised from a polyacrylamide gel, eluted and treated with 0.15 mg/ml trypsin. The digested protein was hydrolyzed with 6 N HCl for 1 hr at 105 °C. Phosphoamino acids were separated by one dimensional thin-layer electrophoresis. The identity of the phosphoryated amino acids was determined by autoradiography and comparison with phosphoamino acid standards.

Figure 3. PKC preferentially phosphorylates wild-type NKX3.1. A, Purified maltose binding protein or NKX3.1 fusion proteins (200 ng) were used as substrates in kinase reactions with PKC (10 ng) and 0.11 μCi [γ-^{32}P]ATP. Following the kinase reactions, samples were electrophoresed on a denaturing 10-20 % gradient polyacrylamide gel. Phosphorylated proteins were identified by autoradiography (top). Western blotting using an anti-maltose binding protein rabbit antiserum (5 μg) was used to control for protein loading (bottom). B, Peptides (30 μg) representing amino acids 43-54 of wild-type (P-WT), R52C (P-R52C) or S48A (P-S48A) NKX3.1 were used in an *in vitro* kinase assay with 10 ng PKC and 0.11 μCi [γ-^{32}P]ATP. Following the kinase reaction, samples were transferred to phosphocelluar discs, washed, and incorporated radioactivity was measured in counts per minute (CPM) by liquid scintillation counting. Amino acid sequences of the peptides are shown below the graph. C, LNCaP cells were transfected with vectors expressing wild-type, R52C or S48A NKX3.1 with an N-terminal FLAG tag. The cells

were treated with R1881 and 48 hr later the cells were exposed to 1 mCi/ml [^{32}P]orthophosphate. Cells were lysed and NKX3.1 was immunoprecipitated with an anti-FLAG antibody. Immunoprecipitants were electrophoresed and radiolabeled proteins were visualized by autoradiography (top). Western blotting using an anti-FLAG antibody (20 μg) was used to control for protein loading (bottom).

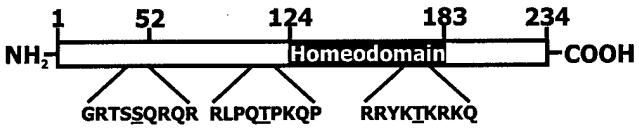
Figure 4. R52C polymorphism affects phosphorylation-regulated DNA binding. Purified fusion proteins (200 ng) were treated with PKC (10 ng) in the presence or absence of cold ATP. Following PKC treatment, the proteins (2, 5, 10 or 25 ng) were used in gel shift assays with a radiolabeled NKX3.1 consensus DNA binding sequence. Protein-bound DNA was separated from free probe by 8 % native PAGE and the results were visualized by autoradiography.

Figure 5. R52C Polymorphism Affects Androgen-Regulated Reporter Gene Expression. CV-1 cells were co-transfected with 250 ng of reporter plasmid, 250 ng of AR expression plasmid and 0 – 2.0 μg wild-type (□) or R52C (■) NKX3.1 expression plasmid. Transfected cells were treated with or without 1 nM dihydrotestosterone. Luciferase activity was measured and standardized according to DHT treatment without co-transfection with NKX3.1 expression plasmid. Luciferase activity was normalized using *Renilla* luciferase plasmid (1 ng).

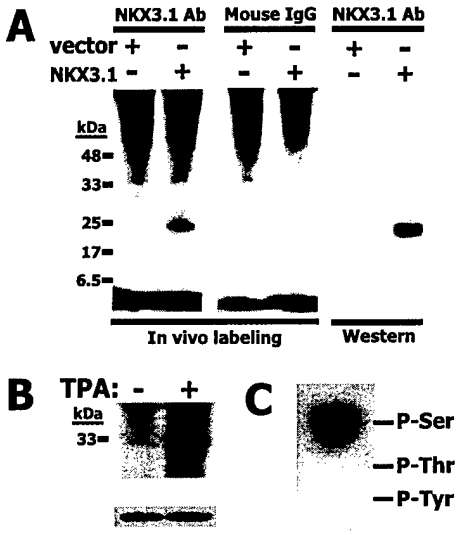
Table 1

NKX3.1 Genotype in Men with Prostate Cancer and Match Controls

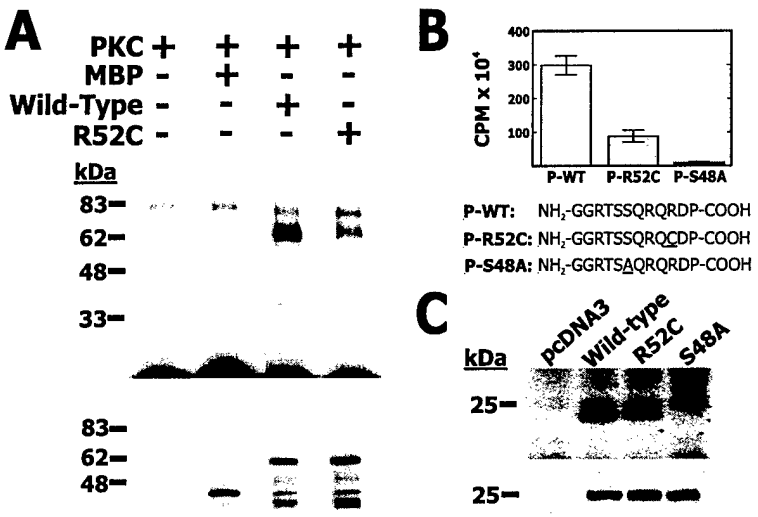
		NKX3.1 Genotype - Nucleotide 154			
RACE	**Case/Control**	**C/C**	**C/T**	**T/T**	**Total**
Caucasian	Control	79 (81.4%)	17	1	97
	Case	81 (85.3%)	13	1	95
	Total	160	30	2	192
African-American	Control	68 (91.9%)	4	2	74
	Case	62 (92.5%)	4	1	67
	Total	130	8	3	141
Total		290 (87.1%)	38	5	333



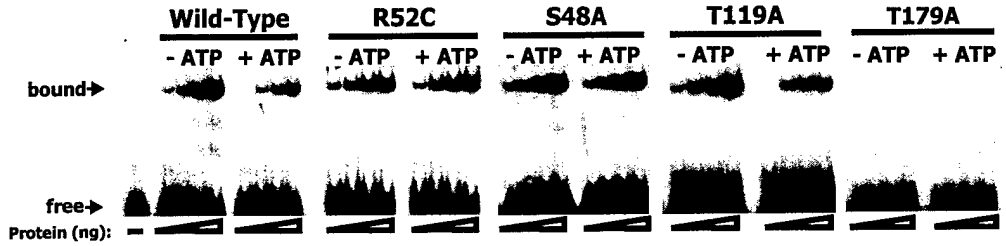
Steadman et al., Figure 1



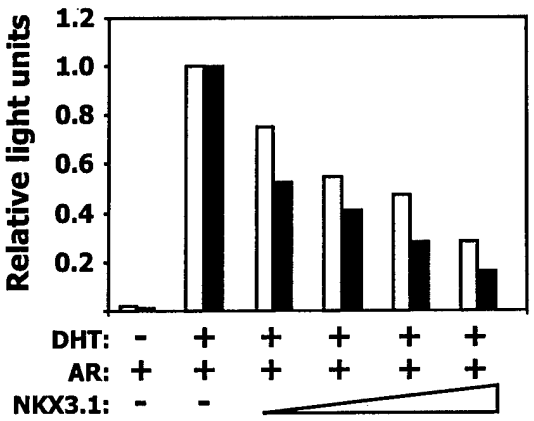
Steadman et al., Figure 2



Steadman et al., Figure 3



Steadman et al., Figure 4



Steadman et al., Figure 5

of dihydrotestosterone in culture medium. Exposure of LNCaP cells to 5-ETA was accompanied by reduced levels of HER-2 protein and the prostate specific antigen (PSA), without affecting the level of androgen receptors. Our data suggested that the ScFv(FRP5)-ETA might be a useful agent for the treatment of human prostate cancer cells with high levels of HER-2 expression.

#4239 OVEREXPRESSION OF THE ADRENOMEDULLIN GENE SIGNIFICANTLY SUPPRESSES ANCHORAGE-INDEPENDENT GROWTH AND TUMORIGENICITY OF PC3 PROSTATE CANCER CELL LINE. Luping Yang, R. Haleem, W. Xiao, and Z. Wang, *Northwestern Univ Med Sch, Chicago, IL*

Adrenomedullin (AM), a recently identified androgen-response gene, is a 52-amino acid peptide which is highly expressed in the epithelium of rat prostate glands. AM is also a multifunctional regulatory peptide in various tissues. However AM's role in mediating androgen action in the prostate has not yet been defined. To elucidate this, a gain-of-function study was performed providing evidence that ectopic overexpression of AM virtually abolishes anchorage-independent growth of PC3 prostatic cancer cells. The inhibition is also supported by the observation that AM-transfected cells show a significant decrease in the rate of cell proliferation as compared to mock-transfected cells and parental cells in monolayer cultures. Further analysis shows that ^{3}H-thymidine incorporation confirms a slower rate of DNA synthesis in AM-transfected cells. The inhibitory effect of AM expression is exclusively shown in PC3 and TSU androgen-independent prostate cancer cells but not in NIH3T3 fibroblasts and UMR106 osteoblasts, suggesting the inhibitory effect of AM is cell-line dependent. AM-transfected cells exhibit a more striking shift to the G1/G0 phase compared to mock-transfected and parental cells, demonstrating AM's ability to arrest cell cycle progression. Moreover, in vivo experiments show that xenograft tumors derived from AM-transfected cells grew profoundly slower than those derived from mock-transfected cells in nude mice. The results of our in vitro and in vivo studies suggest that AM functions as a negative regulator in prostatic tumor progression. The inhibitory effect of AM may have significant clinical implications in the understanding of molecular mechanisms in prostatic tumor suppression.

#4240 β-CATENIN ENHANCES ANDROGEN RECEPTOR SIGNALING. Cristina I Truica, H J Voeller, and E P Gelmann, *Georgetown Univ, Washington, DC*

β-Catenin has pivotal roles in cell-cell adhesion and in signal transduction. Mutations of β-catenin have been found in many cancers and are believed to activate β-catenin signaling. Approximately 5% of primary human prostate tumors have β-catenin mutations. Because β-catenin is a transcriptional activator, we hypothesized that β-catenin could act as a coactivator for the androgen receptor (AR), thereby playing a role in prostate cancer progression. Transient transfection of the LNCaP prostate cell line with two different androgen-responsive reporters, showed that in the presence of androgen, mutant β-catenin increased transcription 3–4-fold compared to control. Similar results were seen in the AR-negative TSU-Prl and PC-3 cell lines in the presence of cotransfected AR. In the absence of AR or androgen, β-catenin had no effect on the expression of the reporter. In the case of the mutant AR found in LNCaP cells (T877A) β-catenin also increased the transcription in the presence of estradiol and hydroxyflutamide. β-Catenin's effect on the activity of the reporters was seen from 0.01 to 10 nM dihydrotestosterone. β-Catenin also relieved the suppression of the antiandrogen bicalutamide (Casodex®) on AR-mediated transactivation. Consistent with the proposed role of E-cadherin in blocking β-catenin's transactivating ability, β-catenin/AR mediated transactivation was antagonized in a dose-dependent manner by transient expression of E-cadherin. β-Catenin was coimmunoprecipitated with AR from LNCaP but not form TSU-Prl cell lysates (AR-), suggesting that the two proteins form a complex in which β-catenin may supply a transactivating domain. Our data suggests a novel mechanism by which β-catenin mutations could contribute to prostate cancer progression, by enhancing androgen receptor signaling, changing the sensitivity of the receptor for ligands and relieving the suppression of AR by antiandrogens.

#4241 IMMUNOHISTOCHEMICAL AND BIOCHEMICAL CHARACTERIZATION OF *NKX3.1* IN NORMAL HUMAN TISSUES AND PROSTATE CANCER. David J Steadman, C Bowen, H J Voeller, and E P Gelmann, *Lombardi Cancer Ctr, Georgetown Univ, Washington, DC*

NKX3.*1* is a prostate-specific homeobox gene that maps to chromosome 8p21, a locus frequently deleted in prostate cancer. In the mouse, *NKX3.1* controls differentiated functions and limits growth of prostate epithelial cells (Bhatia-Gaur et al, *Genes and Develop:* 13:966-977, 1999). Although *NKX3.1* may be a tumor suppressor in humans, the gene is not mutated in human prostate cancer. Immunohistochemical staining of prostate tissues showed expression exclusively in prostate epithelial cells in each of 61 normal samples. As suggested by prior RNA analysis, faint expression was seen in human testis and was localized to seminiferous tubules. Focal expression of NKX3.1 protein was also seen in the ureter, but in no other human tissues. NKX3.1 expression in 31 prostate cancer samples was suppressed in 14, similar in 13 and elevated in 4 compared to adjacent normal cells. Previously, we identified a C to T nucleotide 154 change causing a Cys to Arg mutation at amino acid 52 that was present in 10–14% of the population. The effects of the NKX3.1 mutation were examined by identifying the consensus DNA binding sites for wild-type (WT) and mutant (R52C) NKX3.1. Binding specificity was confirmed by gel mobility shift. Both WT and R52C repressed transcription from a reporter gene downstream from multiple copies of the NKX3.1 binding site. Amino acid 52 is adjacent to a PKC phosporylation site at serine 48 in NKX3.1. *In vitro* phosphorylation studies showed that recombinant R52C phosphorylation was reduced by 60% relative to WT. Phosphorylation of WT resulted in decreased DNA binding affinity, while R52C binding affinity appeared unaltered, presumably due to repressed phosphorylation of the mutant protein. The data suggest the potential for a tumor suppressor function of *NKX3.1*. Furthermore, replacement of arginine with cysteine at position 52 may alter regulation by NKX3.1.

#4242 INHIBITION OF CYCLOOXYGENASE-2 INHIBITS ANGLOGENESIS AND THE GROWTH OF PROSTATE CANCER IN VIVO. X.H. Liu, A. Kirschenbaum, S. Yao, J. F Holland, and A. C Levine, *Mount Sinai Sch of Medicine, New York, NY*

Cyclooxygenase (COX)-2, an inducible enzyme that catalyzes the formation of prostaglandins (PGs) from arachidonic acid, is expressed in prostate cancer and cell lines. Although inhibition of COX-2 activity appears to be effective in colon cancer prevention and treatment, very little attention has been given to COX-2 and its relationship to prostate cancer. In addition, COX-2 has recently been demonstrated to be a stimulator of vascular endothelial growth factor (VEGF) whereby to promote tumor angiogenesis. To evaluate the *in vivo* efficacy of COX-2 inhibition, a selective COX-2 inhibitor, NS398, was tested for its effect on suppression of prostate tumor growth and angiogenesis in nude mice inoculated with PC-3 human prostate cancer cells. Over a 10-week experimental period, NS398 exhibited a sustained inhibition of PC-3 cell tumor growth and a regression of existing tumors. Average tumor surface area at 9 weeks for the NS398 treatment was 0.22 cm^2 versus 2.85 cm^2 from control mice (93 % inhibition, $p < 0.001$). Three out of 14 tumors from drug-treated mice were not palpable. Immunohistochemical examination on the remain tumors suggested that there was no effect of NS398 on tumor proliferation (PCNA), but a significant increase in apoptosis (TUNEL) and a significant decrease in angiogenesis judged by microvessel density (detected by factor-VIII related antigen). VEGF expression was significantly downregulated in the tumors derived from drug-treated mice versus control. These results demonstrate a significant suppression of PC-3 cell tumor growth by inhibition of COX-2 activity in nude mice, and this effect is accomplished by a combination of direct (induction of tumor cell apoptosis) and indirect (inhibition of angiogenesis) actions.

#4243 ^{18}FLUORDEOXYGLUCOSE (FDG) POSITRON EMISSION TOMOGRAPHY (PET) FOR THERAPY MONITORING IN PROSTATE CANCER PATIENTS DURING ANDROGEN WITHDRAWAL. Sebastian Melchior, Jan Fichtner, Rolf Gillitzner, Stefan Dahms, Stefan Both, Peter Benz, Peter Bartenstein, and Joachim Thuroff, *Johannes Gutenberg Univ, Mainz, Germany*

Assessing response to therapy in prostate cancer (CaP) can be challenging because changes in tumor volume are difficult to document and declines in serum prostate specific antigen (PSA) do not always correlate with clinical outcomes. ^{18}FDG-PET is a imaging modality which can be applied to evaluate glucose metabolism in cancer tissue. We investigated the value of ^{18}FDG-PET for monitoring hormonally treated CaP patients. 26 untreated patients with localized (≤T2 NO M0, n=14) or advanced CaP (>T3 No/N+ M0/M+, n=12) were evaluated with ^{18}FDG-PET, magnetic resonance imaging, and serum PSA prior to hormonal treatment and monitored every three months. The results of ^{18}FDG-PET expressed as standard uptake values (SUV) were correlated with tumor stage, grading (Gleason), and cellular proliferation (Ki-67 immunostaining). There was no difference in ^{18}FDG-PET accumulation in organconfined and locally advanced primary tumors. However, ^{18}FDG-accumulation was higher in in poorly differentiated than in low grade cancers. Increased ^{18}FDG uptake was accompanied by a higher proliferation rate. After three months of hormonal therapy intratumoral FDG accumulation was decreased in primary tumors and osseous metastatic lesions in the majority of patients while all showed a decline in serum PSA. ^{18}FDG-PET in CaP patients allows for quantification and changes in tumormetabolism and can be a useful tool for monitoring hormonal therapy. Neoadjuvant or intermittent therapy protocols may be optimized.

#4244 IMMUNOHISTOCHEMICAL LOCALIZATION OF INTERLEUKIN-6 AND ITS RECEPTOR IN PREMALIGNANT AND MALIGNANT PROSTATE TISSUE. A Hobisch, H. Rogatsch, A Hittmair, D Fuchs, G Bartsch jr., H Klocker, G Bartsch, and Z Culig, *Univ of Innsbruck, Innsbruck, Austria*

Interleukin-6 (IL-6) is a multifunctional cytokine that regulates proliferation and secretion in prostate cancer cells. It also regulates expression of prostate-specific proteins by activation of the androgen receptor (Hobisch et al: Cancer Res 58:4640, 1998). To assess the significance of these findings in vivo, we have investigated expression of IL-6 and its receptor in prostate carcinoma by immunohistochemistry. IL-6 immunohistochemistry was performed in 17 frozen prostate cancer specimens and IL-6 receptor immunostaining was evaluated in 21 paraffin-embedded prostate tumor samples. These samples contained adjacent areas of high grade prostate intraepithelial neoplasia (PIN) and benign tissue. In prostate cancer, IL-6 was detected in glandular cells whereas in benign prostate tissue IL-6 immunoreactivity was observed predominantly in basal cells. No IL-6 expression was detected in stromal cells on immunohistochemistry although IL-6 protein was measured in the supernatants obtained from cultured stromal cells by ELISA. IL-6 receptor expression was observed in all tumor specimens investi-